Antifibrotic effects of crocin on thioacetamide-induced liver fibrosis in mice

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Abstract  Liver fibrosis is a major health concern that results in significant morbidity and mortality. Up-to-date, there is no standard treatment for fibrosis because of its complex pathogenesis. Crocin is one of the main nutraceuticals isolated from the stigma of *Crocus sativus* with antioxidant and anti-inflammatory activities. The current study aimed at evaluating the potential antifibrotic activity of crocin against thioacetamide (TAA)-induced liver fibrosis in mice as well as the underlying mechanism using silymarin as a reference antifibrotic product. Crocin at two doses (25 and 100 mg/kg) significantly ameliorated the rise in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, and total bilirubin (TB). Further, the high dose significantly protected against the increase in serum total cholesterol (TC) and triglycerides (TG). These effects were confirmed by light microscopic examinations. Crocin antioxidant activities were confirmed by the observed inhibition of reduced glutathione depletion (GSH), super oxide dismutase (SOD) exhaustion and malondialdehyde (MDA) accumulation in liver tissue. The antifibrotic effects of crocin were explored by assessing fibrosis related gene expression. Administration of crocin (100 mg/kg) hampered expression of tumor growth factor-β (TGF-β), α smooth muscle actin (α-SMA) and collagen 1-α expression and significantly raised gene expression of matrix metalloproteinase-2 (MMP-2). Further, it reduced protein expression of nuclear factor-κB (NF-κB) and cyclooxygenase-2 (COX-2) as assessed immunohistochemically. Thus, it can be concluded that crocin protects against TAA-induced liver fibrosis in mice. This can be ascribed, at least partly, to its antioxidant and anti-inflammatory effects.

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1. Introduction

Liver fibrosis is a major health concern that results in significant morbidity and mortality (Sánchez-Valle et al., 2012). The pathogenesis of liver fibrosis is complex and involves deposition of extracellular matrix (ECM) by activated hepatic stellate cells (HSC) (Reeves and Friedman, 2002). Inflamma-
tion, induced by oxidative stress, is a key event in HSC activation (Greenwel et al., 2000). Several pro-inflammatory cytokines, chemokines and adhesion molecules initiate the activation of HSC (Pinzani and Macias-Barragan, 2010). There is no FDA approved medication for liver fibrosis (Bataller and Brenner, 2005). Several attempts have been made to combat liver fibrosis by inhibiting known molecular targets or pathways that are critically involved in the pathogenesis of fibrogenesis.

Prevention or attenuation of the activation of hepatic stellate and Kupffer cells has been given much attention. Those two cells are responsible for propagation of oxidative and inflammatory responses, with subsequent activation of various fibrogenic mediators (Tacke and Weiskirchen, 2012). Several other targets were proposed that are mainly part of the oxidative or the inflammatory signaling. Molecular targets include nuclear factor-kappa B (NF-κB), tumor growth factor beta (TGF-β) and several inflammatory mediators (Popov and Schuppan, 2009). However, to protect against the development of fibrotic complications associated with the various hepatic injury, a long-term use of the medicaments is necessary. This fact limits the use of chemical products and gives a profound advantage to natural products.

In this regard, natural products have been suggested to combat human diseases including liver fibrosis. Crocin is one such nutraceutical isolated from the stigma of Crocus sativus commonly known as saffron which belongs to family Iridaceae. It is a common dietary colorant carotenoid with 14 free active functional OH groups. It has been reported to possess enormous therapeutic value. Clinically, saffron has shown antidepressant (Moshiri et al., 2006) and anti-Alzheimer activities (Ahkondzadeh et al., 2010) as well as improvement of retinal function (Piccardi et al., 2012). Experimentally, crocin has shown anxiolytic (Pitsikas et al., 2008), antioxidant (El-Beshbishy et al., 2012), neuroprotective (Tamaddonfard et al., 2011), anti-inflammatory (Nam et al., 2010), hematoprotective and genoprotective (Hariri et al., 2011) and gastroprotective (El-Maraghy et al., 2015) activities. Further, crocin has been reported to involve antagonism of TNF-α-induced liver toxicity (Bahashwan et al., 2015) and inhibit stress-induced rise in serum transaminases in rats (Boussabbeh et al., 2016). It protects against chemical insults as patulin poisoning (Boussabbeh et al., 2015). Therefore, the aim of current work was to investigate the potential antifibrotic effect of crocin against thioacetamide-induced liver fibrosis in mice and elucidate the possible underlying mechanisms.

2. Materials and methods

2.1. Chemicals

Crocin and thioacetamide were purchased from Sigma–Aldrich, St. Louis, MO, USA. Other chemicals were of the highest grade commercially available.

2.2. Animals

Experiments were performed on 25–35 g male SWR mice, supplied by the Animal Breeding Laboratory, King Fahd Medical Research Center. Animals were acclimatized for 7 days before the start of experiment in our animal facility. They were maintained on a 12-h light–dark cycle. Room temperature was kept at 22 ± 2°C. Animal handling and experimental protocol were approved by the Unit of Biomedical Ethics, Faculty of Medicine, King Abdulaziz University (Reference # 157-14).

2.3. Experimental protocol

Mice were randomly divided into five groups (8 animals per group) and treated for six consecutive weeks. Group # 1 was considered as control group and given saline i.p. twice weekly. Group # 2 was given thioacetamide (100 mg/kg, i.p.), twice weekly to provoke liver fibrosis. Group # 3 was given both of thioacetamide (100 mg/kg, i.p.) and crocin (25 mg/kg). Group # 4 was given thioacetamide with crocin (100 mg/kg). Group # 5 was given thioacetamide with the positive standard “silymarin” (100 mg/kg). Crocin and silymarin treatments were given by oral gavage every day till day 42. Blood was withdrawn from the retro-orbital plexus. Then, mice were sacrificed. Liver from each animal was rapidly dissected out, washed and homogenized using phosphate-buffered saline (PBS; 50 mM potassium phosphate, pH 7.5) at 4°C, producing a 20% homogenate. In addition to homogenates, representative liver specimens were kept in RNAlater storage solution (Sigma–Aldrich, St. Louis, MO, USA) for RNA extraction. Liver homogenates and liver tissues for RNA extraction were kept at −80°C till time of analyses. Representative liver tissues were kept in 10% formalin-saline for histopathological and immunohistochemical examination.

2.4. Assessment of liver functions

Activities of serum ALT and AST, serum levels of TB, TC and TG were determined colorimetrically using commercially available kits (Biodiagnostic, Cairo, Egypt).

2.5. Histopathological examination

Liver tissues were fixed in 10% neutral buffered formalin for 24 h. This was followed by immersion in tap water and serial dilutions of alcohol. Specimens were cleared in xylene and embedded in paraffin at 56°C in hot air oven for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at 4 μm thickness by slide microtome. Tissue sections were then processed and stained with hematoxylin and eosin for histopathological examinations as previously described (Bancroft and Layton, 2013).
2.6. Assessment of oxidative stress markers

Reduced glutathione (GSH), malondialdehyde (MDA) and protein content as well as superoxide dismutase (SOD) activity were determined in liver homogenates using appropriate commercial kits (Biodiagnostic, Cairo, Egypt).

2.7. Reverse transcriptase–polymerase chain reaction (RT-PCR) analyses of TGF-β, α-SMA, MMP2 and Collagen-1α gene expressions

Total RNA was extracted using PureLink® RNA Mini Kit assay kit. Reverse transcription was carried out using a High-Capacity cDNA Reverse Transcription Kit. Real time PCR was executed using the following primers: TGF-β sense primer, 5′-ACCAACTACTGCTTCAGCTCCACA-3′ and the corresponding antisense primer, 5′-TGTACTGTGTGTCAGGCTCCAAA-3′. α-SMA sense primer, 5′-GGCTCTGGGCTCTGTAAGG-3′ and the corresponding antisense primer, 5′-CTCTTGCTTGGGCTTCATC-3′. MMP2 sense primer, 5′-GAGATCTGCAAACAGGACAT-3′ and the corresponding antisense primer, 5′-GGTTCCTCAGGCTTCAGGTA-3′.

Collagen-1α sense primer, 5′-GGGGTCTAGACATGTT and the corresponding antisense primer, 5′-ACCCATAGGCTATGTACAGTC-3′. Beta-actin was used as reference housekeeping gene with sense primer, 5′-GCCAGACAAATGATCAAGATCA-GATCAT-3′ and the corresponding antisense primer, 5′-ATCTGCTGGAAAGTGGGACA-3′. All the primers were obtained from Invitrogen (Camarillo, CA, USA). Relative gene expressions were evaluated by Quantitative real-time PCR using SYBR Green and Applied Biosystems Step One Real Time PCR System. After 10 min hot start at 95 °C, samples underwent denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min for 45 cycles. Melting curve analysis was performed starting at 60 °C until 95 °C with stepwise temperature elevations. Fluorescent quantitative analysis was carried out with the thermal cycler’s software package to calculate the ΔCt value where the Ct value is the cycle number when the fluorescence curve crossed the baseline value. ΔCt value = target gene Ct value − reference gene Ct value. ΔΔCt = experimental group ΔCt − control group ΔCt. Gene expression in the control group was given a value of unity and extent of gene expression in experimental groups were computed as 2^−ΔΔCt and referred to as relative quantification (RQ).

2.8. Assessment of inflammatory markers

2.8.1. Immunohistochemical detection of cyclooxygenase-2 (COX-2) and NF-κB

Paraffin-embedded tissue sections of 4-μm thickness, were deparaffinized in xylene followed by hydration in graded ethanol solution and heated in citrate buffer (pH 6.0) for 5 min. Sections were then blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 2 h. Slides were then kept overnight at 4 °C with one of the following specific primary antibodies: COX-2 rabbit polyclonal antibody (cat#PA5-23638, Thermo Fischer Scientific), iNOS rabbit polyclonal antibody (cat#RB-9242-P, Thermo Fisher Scientific). Slides were washed with TBS and incubated for 15–20 min with the corresponding secondary antibody. Then, horseradish-peroxidase-conjugated streptavidin solution was added and incubated at room temperature for 15–20 min. The slides were then rinsed with TBS and incubated for 10 min in a solution of 0.02% diaminobenzidine containing 0.01% H2O2. Counter staining was carried out using hematoxylin, and the slides were examined under a light microscope. Immunohistochemical quantification was executed using image analysis software (ImageJ, 1.48a, NIH, USA).

2.8.2. Assessment of IL-1β and TNF-α levels

ELISA kits for IL-1β and TNF-α were obtained from Orgenium Laboratories’ (Vantaa, Finland). The quantitative sandwich immunoassay technique was applied. Briefly, each kit had a specific microplate pre-coated with monoclonal antibody against its analyte. After adding the standards and samples, the analyte was sandwiched by the immobilized antibody and a biotinylated polyclonal antibody specific for it. The later was recognized by a streptavidin–peroxidase conjugate. Unbound materials were then washed away. Thereafter, a peroxidase enzyme substrate was added followed by the stop solution. Finally, color intensity was determined at 450 nm using a microplate reader (ChroMate-4300, FL, USA).

2.9. Statistical analyses

Data were shown as mean ± SD values. Statistical tests were performed using ANOVA followed by Tukey multiple comparisons tests. The 0.05 level of probability or lower were considered the criterion for significance. GraphPad InStat software version 3 (La Jolla, CA, USA) was used to perform statistical tests. Graphs were drawn using GraphPad Prism software version 5 (ISI Software, La Jolla, CA, USA).

3. Results

3.1. Hepatic toxicity

After the TAA-administration, there was a leakage of hepatic enzymes ALT, AST from hepatic cells into the blood, manifested as significant elevation of ALT and AST serum activities compared to the corresponding control group, as shown in Table 1. In addition, TAA administration caused significant increases in TB, TC and TG, compared to the control group. Treatment with crocin (25 and 100 mg/kg) significantly reduced ALT and AST activities, as compared to the corresponding TAA-challenged group. Interestingly, treatment with crocin 25 mg/kg significantly reduced TB concentration but failed to induce any significant changes of TC and TG from the corresponding TAA-challenged group. However, crocin 100 mg/kg has shown significant decreases in TB, TC and TG levels from the corresponding TAA-challenged group. On the other hand, treatment with silymarin normalized the levels of all tested markers that were not statistically different from the corresponding control group.

3.2. Histopathological examination

These hepatotoxicity findings were further confirmed by histopathological examination of representative liver tissues, as shown in Fig. 1. Liver sections from control mice showed...
normal architecture, hepatocyte structure and central vein (Fig. 1A), while TAA-intoxicated mouse livers exhibited focal inflammatory cells infiltration in the hepatic parenchyma surrounding the dilated central vein as well as in the portal area together with diffuse proliferation of kupffer cells between hepatocytes (Fig. 1B). Crocin treatment (25 mg/kg) has shown minimal protective effect as shown in (Fig. 1C) that was similar to sections from TAA-intoxicated liver sections. In contrary, sections taken from a mouse liver treated with crocin (100 mg/kg) and silymarin have provided observable protection with only some degenerative changes (Fig. 1D and E).

3.3. Assessment of oxidative stress

GSH content, MDA level and SOD activity were taken as markers of oxidative status. TAA significantly depleted GSH and caused more than 2-fold increase in MDA levels, compared to the corresponding control group (Fig. 2). These changes were successively restored by silymarin treatment. Besides, treatment with crocin (100 mg/kg) significantly enhanced GSH content and reduced MDA, compared to the corresponding TAA-challenged group. However, Crocin (25 mg/kg) treatment failed to show any significant change of MDA level, compared to TAA-challenged group. A similar pattern of biological activity was observed SOD activity that was significantly decreased to about 13% of the control by the TAA challenge. On the other hand, treatment with crocin (25 and 100 mg/kg) and silymarin significantly increased its activity, reaching 55%, 71% and 80% of the control activity, respectively (Fig. 2).

3.4. Assessment of liver fibrosis

Gene expression analyses of fibrosis-related genes were carried out to evaluate the impact of crocin treatment on the extent of hepatic fibrosis. As shown in (Fig. 3), TAA-challenged mice showed significant up-regulation of TGF-β1, α-SMA and collagen-1 genes to 8.9, 8.4 and 10.1 folds, respectively. Meanwhile, MMP-2 gene expression was significantly decreased by about 87% of the control value. It is worthy to note that both crocin (100 mg/kg) and silymarin treatments significantly protected against such changes of gene expression, showing no significant differences from the corresponding control. However, treatment with crocin (25 mg/kg) was unable to demonstrate any significant difference from the corresponding TAA-challenged group.

3.5. Assessment of inflammatory markers

Expression of NF-κB activated subunit p65 in liver tissues and the proinflammatory enzyme COX-2 was assessed by immuno-histochemical staining. As shown in (Fig. 4A), minimal immunostaining for both NF-κB and COX-2 was observed in sections from control group. On contrary, TAA elevated the expression of these markers as shown by the intense brown staining (Fig. 4B), especially around the central veins which

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT U/L</th>
<th>AST U/L</th>
<th>TB mg/dL</th>
<th>TC mg/dL</th>
<th>TG mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.02 b ± 4.23</td>
<td>13.25 b ± 2.04</td>
<td>0.23 b ± 0.084</td>
<td>58.98 b ± 3.06</td>
<td>73.13 b ± 3.79</td>
</tr>
<tr>
<td>Thioacetamide (TAA; 100 mg/kg)</td>
<td>129.13 a ± 17.09</td>
<td>95.54 a ± 3.53</td>
<td>1.20 a ± 0.249</td>
<td>126.53 a ± 6.56</td>
<td>156.90 a ± 8.14</td>
</tr>
<tr>
<td>TAA + crocin 25 mg/kg</td>
<td>52.86 a,b ± 0.43</td>
<td>47.28 a,b ± 3.22</td>
<td>0.83 a,b ± 0.29</td>
<td>124.00 a ± 6.43</td>
<td>153.76 a ± 7.97</td>
</tr>
<tr>
<td>TAA + crocin 100 mg/kg</td>
<td>28.00 a,b ± 0.50</td>
<td>29.14 a,b ± 5.18</td>
<td>0.36 a,b ± 0.129</td>
<td>69.45 a,b ± 3.60</td>
<td>86.12 a,b ± 4.46</td>
</tr>
<tr>
<td>TAA + silymarin 100 mg/kg</td>
<td>22.17 a b ± 5.24</td>
<td>19.46 a,b ± 4.32</td>
<td>0.29 a,b ± 0.103</td>
<td>64.29 a,b ± 3.33</td>
<td>79.71 a,b ± 4.13</td>
</tr>
</tbody>
</table>

n = 6. Statistical analysis was carried out by a one-way ANOVA followed by Tukey’s post hoc test.

a Statistically significant from the corresponding control at p < 0.05.
b Statistically significant from TAA-treated group at p < 0.05.

**Figure 1** Representative photomicrographs of liver sections stained by H&E (×100): (A) Control section with normal histological structure of the central vein and surrounding hepatocytes, (B) TAA-challenged group with focal inflammatory cells infiltration in the hepatic parenchyma surrounding the dilated central vein as well as in the portal area associated with diffuse kupffer cells proliferation in between the hepatocytes, (C) treatment with crocin 25 mg/kg showing focal inflammatory cells infiltration in the parenchyma surrounding the central vein with diffuse kupffer cells proliferation in between the hepatocytes, (D) treatment with crocin 100 mg/kg in which hepatocytes show degenerative change associated with focal inflammatory cell infiltration in the hepatic parenchyma. (E) treatment with silymarin 100 mg/kg showing degenerative change was diffuse manner all over the hepatocytes.
correlates with the pathological centrilobular changes detected by H&E staining. Crocin (25 mg/kg) treatment did not alleviate such changes as shown in (Fig. 4C). However, treatment with crocin (100 mg/kg) and silymarin prevented these alterations to a large extent (Fig. 4D and E), showing only diffuse brown staining which again corresponds to the diffuse degeneration observed in H&E staining.

The previous effects of crocin treatment were further confirmed by assessing IL-1β and TNF-α using ELISA technique. As shown in (Fig. 5A and B), TAA caused significant 4.8 and 6.4 fold-increases in IL-1β and TNF-α, respectively. Treatment with crocin (25 mg/kg) significantly reduced their levels by 46.6% and 32.8% of the corresponding TAA group, respectively. The effect of crocin (100 mg/kg) was more pronounced, being able to cause significant reductions of IL-1β and TNF-α by 61.6% and 71.7% of the corresponding TAA group, respectively. It is worth mentioning that crocin (100 mg/kg) and silymarin did not show any significant difference of both parameters from the corresponding control group.

4. Discussion

Liver fibrosis is a major health distress that causes significant morbidity and mortality. Up to date, there is no standard treatment for fibrosis because of its complex pathogenesis. Inflammation, induced by oxidative stress, is a key factor in HSC activation (Greenwel et al., 2000) which in turn involved
in deposition of ECM (Reeves and Friedman, 2002). Pathological inflammation has been suggested to activate transcription of pro-apoptotic genes and damage hepatocytes. Also, pro-inflammatory cytokines have been shown to exacerbate oxidative damage of the liver (Mohamed et al., 2016). There is no FDA approved medication for liver fibrosis (Bataller and Brenner, 2005). Crocin is one of the main nutraceutical isolated from the stigma of *C. sativus* with antioxidant, anti-apoptotic and anti-inflammatory activities. Therefore, the current study aimed at evaluating the potential antifibrotic activities of crocin against thioacetamide-induced liver fibrosis in mice as well as the underlying mechanism.

The hepatotoxin TAA has been utilized in a multiplicity of studies evaluating the core mechanisms of liver fibrogenesis (Schnur et al., 2004; Sáez-Lara et al., 2006; Kornek et al., 2006). In comparison to other experimental models, TAA-induced liver fibrosis is intimately like to alcoholic liver fibrogenesis with similar histological and metabolic alterations commonly detected in the livers of afflicted humans (Nozu et al., 1992). In the present study, treatment with crocin (25 and 100 mg/kg) or silymarin significantly preserved hepatocyte integrity as indicated by reduced plasma activities of ALT and AST. It is noteworthy that the higher dose of crocin also improved the metabolic function as marked by plasma total bilirubin, cholesterol and triglycerides. These observations were confirmed histologically as crocin (100 mg/kg) mitigated hepatic injury and pericentral fibrosis and inflammatory cells induction. In addition, TAA-induced oxidative stress in hepatic tissues was alleviated by crocin treatment. This was evidenced by enhanced GSH content and reduced lipid peroxidation. Notably, crocin at 100 mg/kg was superior to its lower dose (25 mg/kg) in preserving SOD activity. The observed preserved integrity of hepatocyte membranes by crocin can be explained by its well-known antioxidant properties (El-Beshbishy et al., 2012). This antioxidant activity was in accordance with the previous work of Hemshekhar et al. (2012); in which crocin ameliorated the altered antioxidant status via enhancing GSH, SOD, CAT and GST in a rat model of arthritis.

Liver fibrosis is characterized by quantitative and qualitative amendments of hepatic ECM. The fundamental cellular event is HSC activation. Upon stimulation, quiescent HSCs are differentiated into myofibroblast-like cells with increased proliferation, accumulation of ECM – and expression of α-
SMA, (Friedman, 2000). As a result, accumulation of collagen in the liver is considered as a mark of fibrosis (Bissell et al., 1990). In the current study, the antiinflammatory activity of crocin was principally examined by assessing gene expression of fibrosis-related genes. Up-regulation of α-SMA and collagen I-1α genes is a fundamental step that conveys HSCs activation and liver fibrosis (Bataller and Brenner, 2005). The up-regulation of TGF-β as well as down-regulation of MMP-2 indicates the pathogenesis of fibrosis (Knittel et al., 2000; Guo and Friedman, 2007). Crocin (100 mg/kg) significantly inhibited over-expression of collagen-1α, α-SMA, TGF-β as well as the down-regulation of MMP-2 expression. It is worth mentioning that crocin (25 mg/kg) was unable to ameliorate TAA-induced modulation of fibrosis-related gene expression.

Subsequently, the antiinflammatory mechanism of crocin was evaluated by studying different markers of inflammation. Assessment of inflammatory markers revealed that TAA induced a significant increase in hepatic NF-κBp65 and COX-2 expression together with significant elevation in tissue levels of the TNF-α and IL-1β. This indicates amplified inflammatory responses induced by TAA challenge. In this regard, kinases activation in NF-κB pathway is connected to oxidative stress (Baeuerle and Baichwal, 1997). However, antiangiogenic activities cannot be excluded based on those observed with crocetin (Umigai et al., 2012). ROS are shown to cause prolonged NF-κB DNA binding activity (Ripple et al., 1999). This resulted in enhanced production of proinflammatory cytokines such as TNF-α and IL-6 that are implicated in fibrosis (Racanelli and Reherrman, 2006). NF-κB also stimulates the expression of enzymes whose products participate in the pathogenesis of inflammation, including COX-2 (Pahl, 1999). COX-2 activity leads to high levels of eicosanoids resulting in cellular inflammation and necrosis (Hu, 2003). This was in line with the previous study of (Orfila et al., 2005), in which NF-κB induction during liver injury has been reported in hepatocytes in a model of CCl4-induced hepatotoxicity in rats. Thus, elevated levels of NF-κB promote the secretion of inflammatory and chemotactic factors in hepatocytes and worsen hepatic inflammation and fibrosis (Luedde and Schwabe, 2011). In the present study, treatment with crocin significantly reduced the expression of NF-κB and consequently inhibited the downstream inflammatory cascade. This is evidenced by decreasing the expression of COX-2 and levels of TNF-α and IL-1β. Thus, crocin proved to have reasonable antiinflammatory activity. In conclusion, the present study indicates the potential antifibrotic activity of crocin, which can be attributed – at least partly – by its antioxidant and antiinflammatory actions.

References


