

ORIGINAL ARTICLE

# Umbelliferone Ameliorates CCl<sub>4</sub>-Induced Liver Fibrosis in Rats by Upregulating PPAR $\gamma$ and Attenuating Oxidative Stress, Inflammation, and TGF- $\beta$ 1/Smad3 Signaling

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**Abstract**—Umbelliferone (UMB) is a natural coumarin that has diverse biological activities. However, its potential to protect against liver fibrosis has not been reported yet. This study aimed to investigate the protective effect of UMB against carbon tetrachloride (CCl<sub>4</sub>)-induced liver fibrosis in rats. Rats received CCl<sub>4</sub> and UMB for 8 weeks and samples were collected for analyses. CCl<sub>4</sub> induced a significant increase in serum levels of liver function markers and pro-inflammatory cytokines. Treatment with UMB significantly ameliorated liver function markers and pro-inflammatory cytokines and prevented CCl<sub>4</sub>-induced histological alterations. CCl<sub>4</sub> promoted significant upregulation of  $\alpha$ -smooth muscle actin (SMA), collagen I, collagen III, NF- $\kappa$ B p65, TGF- $\beta$ 1, and p-Smad3. Masson's trichrome staining revealed a significant fibrogenesis in CCl<sub>4</sub>-induced rats. Treatment with UMB suppressed TGF- $\beta$ 1/Smad3 signaling and downregulated  $\alpha$ -SMA, collagen I, collagen III, and NF- $\kappa$ B p65. In addition, UMB diminished malondialdehyde and nitric oxide levels, boosted reduced glutathione and antioxidant enzymes, and upregulated the expression of PPAR $\gamma$ . In conclusion, our results demonstrated that UMB prevented CCl<sub>4</sub>-induced liver fibrosis by attenuating oxidative stress, inflammation, and TGF- $\beta$ 1/Smad3 signaling, and upregulating PPAR $\gamma$ . Therefore, UMB may be a promising candidate for preventing hepatic fibrogenesis, given that further research is needed to delineate the exact molecular mechanisms underlying its antifibrotic efficacy.

**KEY WORDS:** fibrosis; 7-hydroxycoumarin; oxidative stress; inflammation; TGF- $\beta$ 1; PPAR $\gamma$ .

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## INTRODUCTION

Chronic liver disease is a progressive destruction and regeneration of the liver tissue leading to hepatic fibrosis and cirrhosis. It represents a major health problem and a cause of morbidity and mortality [1]. Liver fibrosis is a ubiquitous wound-healing response to chronic tissue injury caused by hepatitis, biliary obstruction, nonalcoholic fatty liver disease, metabolic liver disease, and others [2]. It is a complex and multifactorial process associated with increased deposition of collagen-rich extracellular matrix (ECM). If left untreated, fibrosis can develop into cirrhosis,

and consequently hepatocellular carcinoma (HCC), liver failure, and death [3]. Hepatic stellate cells (HSCs) play the central role in fibrogenesis of the liver. These cells are located between hepatocytes and endothelial cells of the sinusoids and being activated during liver injury [4]. Activated HSCs acquire myofibroblast-like phenotypes and become the source of activated myofibroblasts and portal fibroblasts. Consequently, the production and deposition of fibrous ECM proteins increase, leading to liver fibrosis [5]. The fibrotic activity of HSCs could be promoted by different factors, including inflammatory cytokines and reactive oxygen species (ROS) [6, 7]. In addition, the transforming growth factor beta 1 (TGF- $\beta$ 1)/Smad3 signaling was evidenced to induce liver fibrosis. Activated TGF- $\beta$ 1/Smad3 signaling induces the synthesis and production of ECM rich in collagen I and III [8, 9]. Therefore, attenuation of TGF- $\beta$ 1/Smad3 signaling and suppression of oxidative stress and inflammation represent an efficient strategy to inhibit fibrogenesis.

Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) is a ligand-activated nuclear receptor that participates in many biological processes, mainly adipogenesis and metabolism [10]. In addition to its metabolic functions, it possesses a potent anti-inflammatory efficacy [11], and can induce the expression of antioxidant enzymes [12] and suppress TGF- $\beta$ 1/Smad3 signaling [13]. Previously, we have reported that upregulation of PPAR $\gamma$  can protect against hepatocarcinogenesis [14], and liver injury induced by cyclophosphamide [15–18], methotrexate [19, 20], and azoxymethane [21]. In addition, multiple studies have revealed the role of PPAR $\gamma$  in ameliorating hepatic fibrosis [13, 22, 23].

Umbelliferone (UMB) is a natural product found in plants of the *Rutaceae* and *Umbelliferae* families. It is also known as 7-hydroxycoumarin and commonly used as a sunscreen agent [24]. UMB possesses several pharmacological activities, including antioxidant, anti-inflammatory, antidiabetic, and antitumor effects [24, 25]. UMB prevented cyclophosphamide hepatotoxicity by upregulating PPAR $\gamma$  as we have recently reported [17]. In a rat model of hyperammonemia, UMB protected the brain and liver against the deleterious effects of excess ammonia [26]. In addition, UMB has been recently demonstrated to prevent liver injury in *db/db* mice [27]. In these studies, the effects of UMB were mediated mainly *via* suppression of oxidative stress and inflammation. These beneficial effects make UMB a good candidate for the protection against liver fibrosis. Therefore, we investigated the efficacy of UMB to ameliorate fibrosis induced by carbon tetrachloride (CCl<sub>4</sub>) in rats, pointing to its ability to modulate TGF- $\beta$ 1/

Smad3 signaling and PPAR $\gamma$  expression. CCl<sub>4</sub> is frequently used to induce liver injury [28–31] and fibrogenesis in rodents [6, 32].

## MATERIALS AND METHODS

### Reagents and Chemicals

UMB, CCl<sub>4</sub>, malondialdehyde (MDA), reduced glutathione (GSH), thiobarbituric acid, pyrogallol, Griess reagent, trichloroacetic acid, and 5,5'-dithio-bis-[2-nitrobenzoic acid] were purchased from Sigma (USA). Assay kits for aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyltransferase ( $\gamma$ GT), bilirubin, and albumin were supplied by Spinreact (Girona, Spain), and the Bradford protein assay kit was purchased from BioBasic (Markham, ON, Canada). Primary antibodies for TGF- $\beta$ 1, p-Smad3, Smad3, PPAR $\gamma$ , nuclear factor-kappaB (NF- $\kappa$ B) p65, and  $\beta$ -actin, and secondary antibodies were supplied by Novus Biologicals (Centennial, CO, USA). Assay kits for tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin (IL)-6 were supplied by R&D Systems (USA), and TRIzol was purchased from Invitrogen (USA). All other chemicals were obtained from standard commercial supplies.

### Experimental Animals and Treatments

Male Wistar rats, weighing about 160–180 g, obtained from the National Institute of Ophthalmology (Egypt) were included in this study. The animals were housed at normal temperature ( $23 \pm 2$  °C) and 12-h light/dark cycle. A standard diet and water were provided *ad libitum*, and the experimental protocol and all animal procedures were approved by the Institutional Animal Ethics Committee of Beni-Suef University (Egypt).

Thirty rats were divided into five groups ( $n = 6$ ) as follows (Fig. 1):

- Group I (Control): received intraperitoneal (i.p.) injection of 1 ml/kg olive oil twice/week and 1% carboxymethyl cellulose (CMC) by daily oral gavage for 8 weeks
- Group II (CCl<sub>4</sub>): received i.p. injection of 1 ml CCl<sub>4</sub>/olive oil (1:1 v/v) twice/week [33] and 1% CMC by daily oral gavage for 8 weeks
- Group III (CCl<sub>4</sub> + 25 mg UMB): received i.p. injection of CCl<sub>4</sub> as group II and 25 mg/kg UMB dissolved in 1% CMC daily by oral gavage for 8 weeks

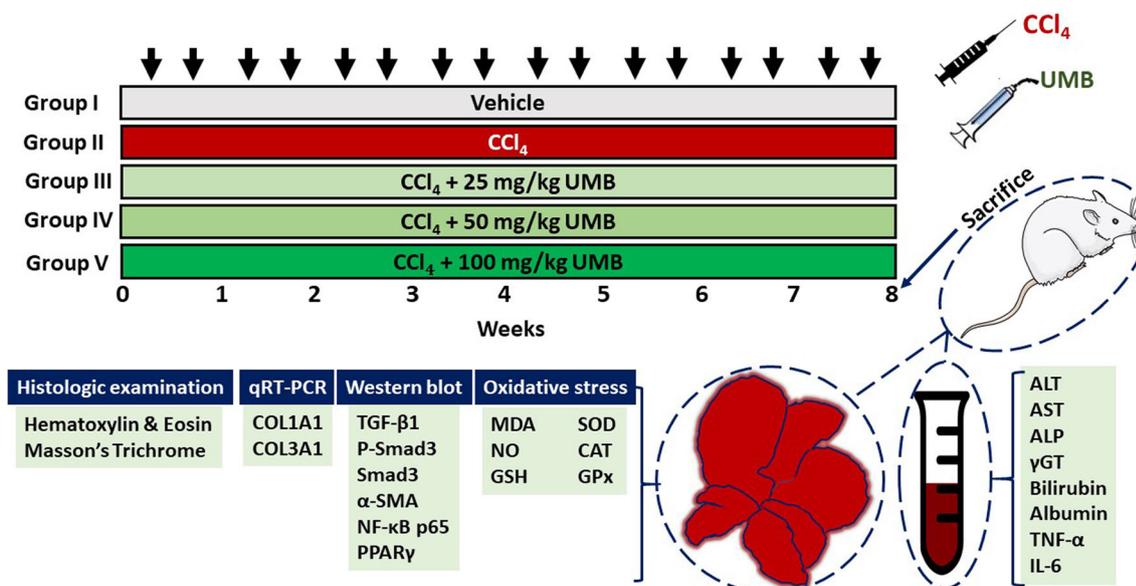


Fig. 1. A schematic diagram showing the experimental design.

Group IV (CCl<sub>4</sub> + 50 mg UMB): received i.p. injection of CCl<sub>4</sub> as group II and 50 mg/kg UMB dissolved in 1% CMC daily by oral gavage for 8 weeks

Group V (CCl<sub>4</sub> + 100 mg UMB): received i.p. injection of CCl<sub>4</sub> as group II and 100 mg/kg UMB dissolved in 1% CMC daily by oral gavage for 8 weeks

The doses of UMB used in this study were selected based on our previous studies reporting its antioxidant and hepatoprotective effects *in vivo* [17, 26], and was regularly adjusted based on the body weight changes. All rats were sacrificed under anesthesia at the end of 8 weeks. Blood samples were collected for serum separation, and liver was excised and washed in cold phosphate-buffered saline (PBS). Pieces from the liver were fixed in 10% neutral buffered formalin, and other samples were homogenized in cold PBS (10% *w/v*), while others were kept frozen at -80 °C for gene and protein expression analysis.

#### Assay of Liver Function Markers and Pro-inflammatory Cytokines

Serum levels of ALT, AST, ALP,  $\gamma$ GT, bilirubin, and albumin were measured using commercially available kits (Spinreact, Girona, Spain). TNF- $\alpha$  and IL-6 were assayed in serum of control and experimental rats using specific ELISA kits (R&D Systems, USA).

#### Assay of MDA, Nitric Oxide, and Antioxidants

MDA [34] and nitric oxide (NO) [35] levels were measured in the liver homogenate (10% *w/v* in PBS). GSH, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were determined according to the methods of Beutler et al. [36], Marklund and Marklund [37], Aebi [38], and Matkovic et al. [39], respectively.

#### Histological Examination

The liver samples were fixed in 10% neutral buffered formalin for 48 h and then processed for the preparation of paraffin blocks. Five-micrometer sections were cut using a rotatory microtome and then stained with hematoxylin and eosin (H&E) and Masson's trichrome (MT) and examined using a light microscope. The MT-stained areas in the liver sections were quantified using ImageJ (version 1.32j, NIH, USA).

#### Gene Expression Analysis

The effect of UMB on collagen I (COL1A1) and collagen III (COL3A1) mRNA expression levels in the liver of CCl<sub>4</sub>-intoxicated rats was evaluated using quantitative reverse transcriptase real-time polymerase chain reaction (qRT-PCR) as previously described [19]. Total RNA was isolated from the liver samples using TRIzol. The isolated RNA was quantified, and samples with

A260/A280 of 1.8 or more were used for reverse transcription. Amplification of the cDNA was carried out by SYBR Green and the following primers: COL1A1 F: 5'-GTAC ATCAGCCCAAACCCCA-3' and R: 5'-CAGGATCG GAACCTTCGCTT-3', COL3A1 F: 5'-AGGGCAGG GAACAACCTGATG-3' and R: 5'-GGTCCCACATTGCA-CAAAGC-3', and  $\beta$ -actin F: 5'-AGGAGTACGATG AGTCCGGC-3' and R: 5'-CGCAGCTCAGTAACAGTC CG-3'. The obtained data were analyzed using the  $2^{-\Delta\Delta Ct}$  method [40] and normalized to  $\beta$ -actin.

### Western Blotting

The liver samples were homogenized in RIPA buffer with proteinase inhibitors and centrifuged at 10,000 rpm, and the clear supernatant was collected. The Bradford protein assay kit was used to determine protein concentration, and 40  $\mu$ g proteins was subjected to 10% SDS-PAGE followed by transfer to nitrocellulose membranes. The membrane was blocked for 1 h at room temperature and incubated with antibodies for TGF- $\beta$ 1, p-Smad3, Smad3, PPAR $\gamma$ , NF- $\kappa$ B p65, and  $\beta$ -actin overnight at 4 °C. The membranes were incubated with the secondary antibodies, developed using the enhanced chemiluminescence kit (BIO-RAD, USA), and the obtained bands were quantified using ImageJ (version 1.32j, NIH), and the results were presented as percent of control.

### Statistical Analysis

The results were analyzed using GraphPad Prism5 (GraphPad software, San Diego, CA, USA) and expressed as mean  $\pm$  standard error of the mean (SEM). Different parameters among the experimental groups were compared by one-way ANOVA, followed by Tukey's *post hoc* test. The difference was considered significant at  $P < 0.05$ .

## RESULTS

### UMB Inhibits CCl<sub>4</sub>-Induced Functional and Histological Alterations in the Liver of Rats

The circulating levels of liver function markers were measured to determine the protective effect of UMB on CCl<sub>4</sub>-induced liver injury. CCl<sub>4</sub>-administered rats exhibited a significant increase in serum ALT (Fig. 2a;  $P < 0.001$ ), AST (Fig. 2b;  $P < 0.001$ ), ALP (Fig. 2c;  $P < 0.001$ ), and  $\gamma$ GT (Fig. 2d;  $P < 0.001$ ). Concurrent treatment with UMB for 8 weeks notably ameliorated serum levels of the liver function markers in CCl<sub>4</sub>-induced

rats. The high dose of UMB (100 mg/kg) produced a significant ( $P < 0.001$ ) reduction in serum AST levels when compared with the 25 mg/kg dose (Fig. 2b). Serum bilirubin was increased significantly ( $P < 0.001$ ) in CCl<sub>4</sub>-induced rats and remarkably decreased in 25, 50, and 100 mg/kg UMB-treated groups (Fig. 2e). In contrast, serum albumin was decreased ( $P < 0.001$ ) in CCl<sub>4</sub>-induced rats as represented in Fig. 2f. UMB significantly improved serum albumin levels when administered at 25 ( $P < 0.05$ ), 50 ( $P < 0.001$ ), and 100 ( $P < 0.001$ ) mg/kg into CCl<sub>4</sub>-induced rats.

The hepatoprotective effect of UMB was further confirmed by the histopathologic examination (Fig. 3). H&E-stained sections of control rats showed normal histological structure of the liver (Fig. 3a). CCl<sub>4</sub>-induced rats exhibited multiple alterations, including focal necrosis, steatosis of hepatocytes, cytoplasmic vacuolization of hepatocytes, Kupffer cell activation, and fibroblast proliferation (Fig. 3b, c). On the other hand, CCl<sub>4</sub>-induced rats that received 25 (Fig. 3d), 50 (Fig. 3e), and 100 mg/kg UMB (Fig. 3f) showed a notable improvement in the histological appearance of the liver with slight cytoplasmic vacuolations (Fig. 3d, f). The histopathological lesions in the liver of all experimental groups are summarized in Table 1.

### UMB Prevents CCl<sub>4</sub>-Induced Liver Fibrosis in Rats

Fibrosis in the liver of CCl<sub>4</sub>-intoxicated rats was evaluated using MT staining and assessment of the expression levels of  $\alpha$ -SMA and collagen. Image analysis of the MT-stained liver sections of CCl<sub>4</sub>-induced rats revealed an increase in ECM deposition ( $P < 0.001$ ) when compared with the control rats (Fig. 4a and 5b). Oral supplementation of all UMB doses for 8 weeks reduced ECM deposition significantly ( $P < 0.001$ ).

Liver  $\alpha$ -SMA was remarkably ( $P < 0.001$ ) upregulated in CCl<sub>4</sub>-intoxicated rats when compared with the control group as represented in Fig. 4c. The mRNA abundance of COL1A1 (Fig. 4d) and COL3A1 (Fig. 4e), determined by qRT-PCR, showed a notable ( $P < 0.001$ ) upregulation in CCl<sub>4</sub>-induced rats. In contrast, CCl<sub>4</sub>-intoxicated rats that received different doses of UMB exhibited a significant decrease in the expression levels of  $\alpha$ -SMA, COL1A1, and COL3A1.

### UMB Downregulates Hepatic TGF- $\beta$ 1/Smad3 Signaling in CCl<sub>4</sub>-Intoxicated Rats

TGF- $\beta$ 1 protein expression was significantly upregulated in the liver of CCl<sub>4</sub>-intoxicated rats as

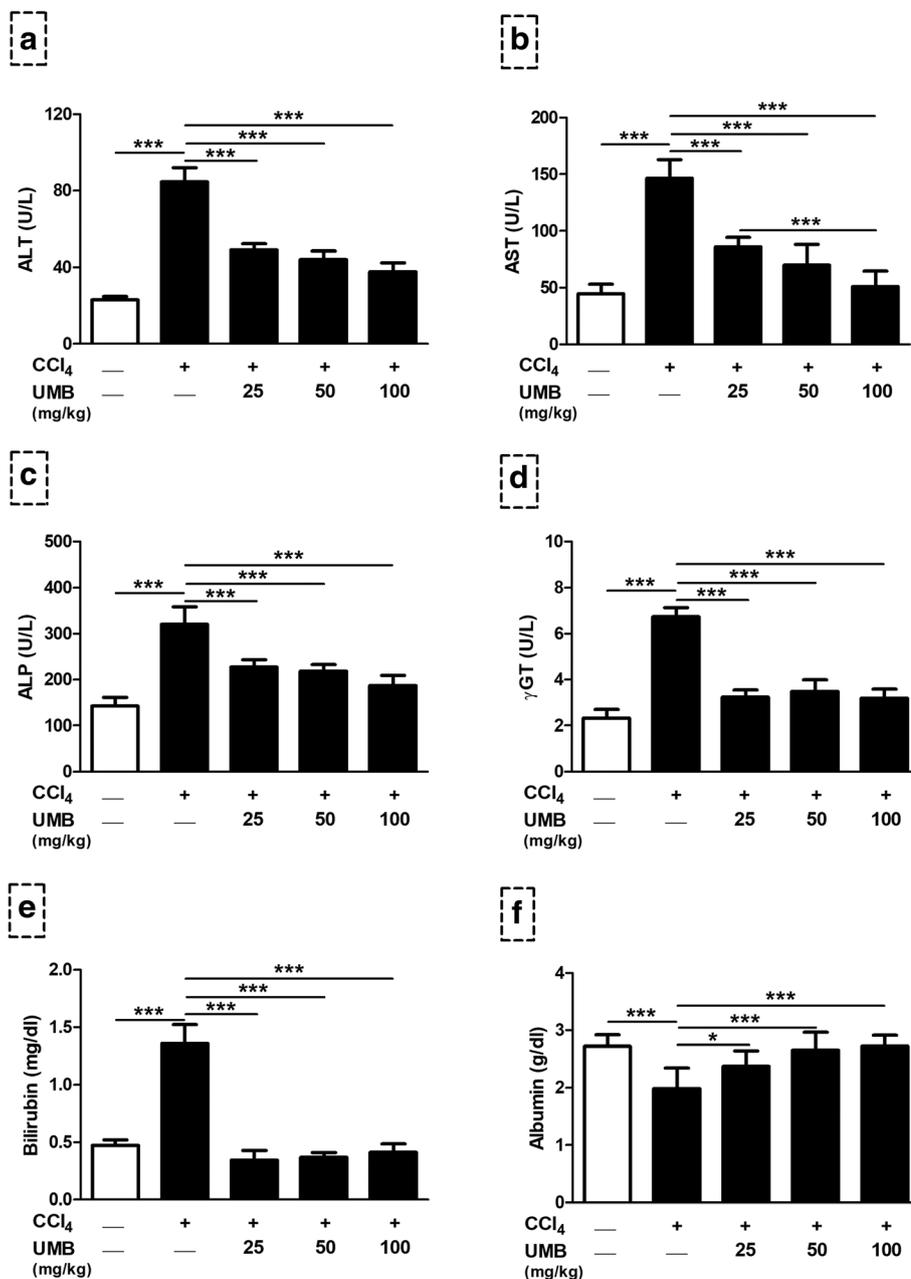
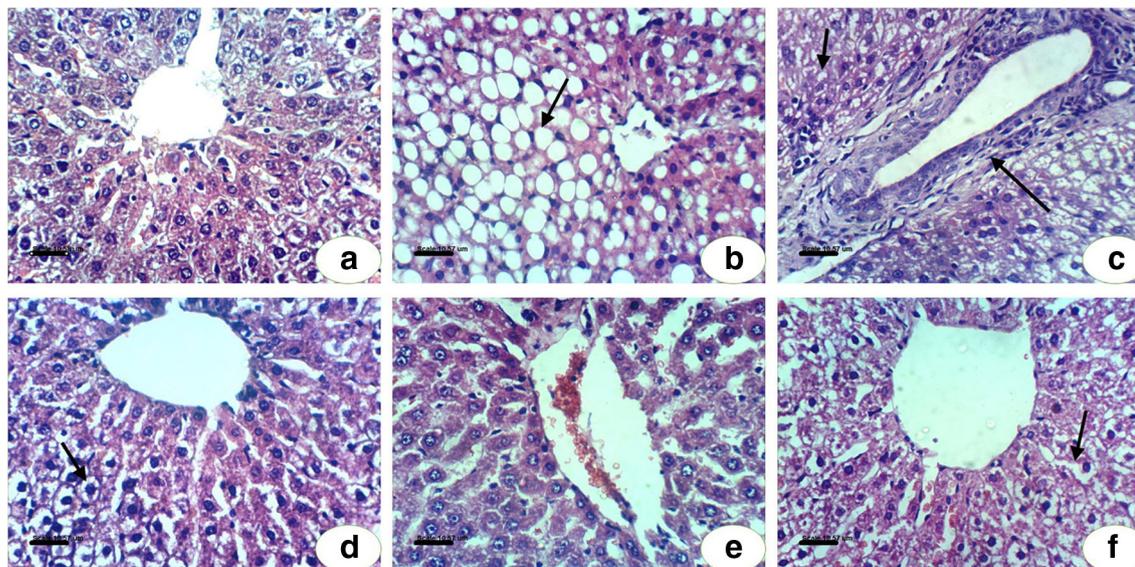


Fig. 2. Umbelliferone inhibits CCl<sub>4</sub>-induced liver injury in rats. Treatment with UMB ameliorated serum levels of a ALT, b AST, c ALP, d  $\gamma$ GT, e bilirubin, and f albumin in CCl<sub>4</sub>-induced rats. Data are expressed as mean  $\pm$  SEM,  $n = 6$ . \* $P < 0.05$  and \*\*\* $P < 0.001$ .

represented in Fig. 5a, b. Similarly, Smad3 phosphorylation was remarkably ( $P < 0.001$ ) increased in CCl<sub>4</sub>-intoxicated rats (Fig. 5a, c). Treatment with 25, 50, and 100 mg/kg UMB suppressed TGF- $\beta$ 1 expression and Smad3 phosphorylation.

#### UMB Attenuates CCl<sub>4</sub>-Induced Oxidative Stress in the Liver of Rats

MDA, a marker of lipid peroxidation, was increased significantly ( $P < 0.001$ ) in the liver of rats that received CCl<sub>4</sub> as represented in Fig. 6a. Similarly, NO was



**Fig. 3.** Umbelliferone prevents  $\text{CCl}_4$ -induced histological alterations in the liver of rats. H&E-stained liver sections in **a** control rats showing normal histological structure of hepatic lobule; **b** and **c**  $\text{CCl}_4$ -induced rats showing steatosis of hepatocytes [**b**; arrow], cytoplasmic vacuolization of hepatocytes [**c**; short arrow], and fibroblast proliferation in the portal triad [**c**; long arrow]; and  $\text{CCl}_4$ -induced rats treated with 25 mg/kg (**d**), 50 mg/kg (**e**), and 100 mg/kg (**f**) showing slight cytoplasmic vacuolization (arrow). ( $\times 400$ ).

significantly ( $P < 0.001$ ) elevated in the liver of rats received  $\text{CCl}_4$  (Fig. 6b). Treatment with 25, 50, and 100 mg/kg UMB produced a significant decrease in hepatic MDA ( $P < 0.01$ ;  $P < 0.001$ ;  $P < 0.001$ ) and NO levels ( $P < 0.001$ ;  $P < 0.001$ ;  $P < 0.001$ ).

On the other hand, the GSH content was reduced significantly ( $P < 0.001$ ) in  $\text{CCl}_4$ -induced rats (Fig. 6c). UMB supplementation significantly increased the GSH content when administered at 25 ( $P < 0.05$ ), 50 ( $P < 0.05$ ), and 100 mg/kg body weight ( $P < 0.01$ ). Activities of SOD (Fig. 6d), CAT (Fig. 6e), and GPx (Fig. 6f) were significantly ( $P < 0.001$ ) declined in  $\text{CCl}_4$ -induced rats. The 25 mg/kg UMB did not induce a significant improvement in SOD activity, whereas it enhanced the activity of both CAT ( $P < 0.05$ ; Fig. 6e) and GPx

( $P < 0.001$ ; Fig. 6f). The higher UMB doses (50 and 100 mg/kg) enhanced the activity of SOD, CAT, and GPx significantly in the liver of  $\text{CCl}_4$ -induced rats.

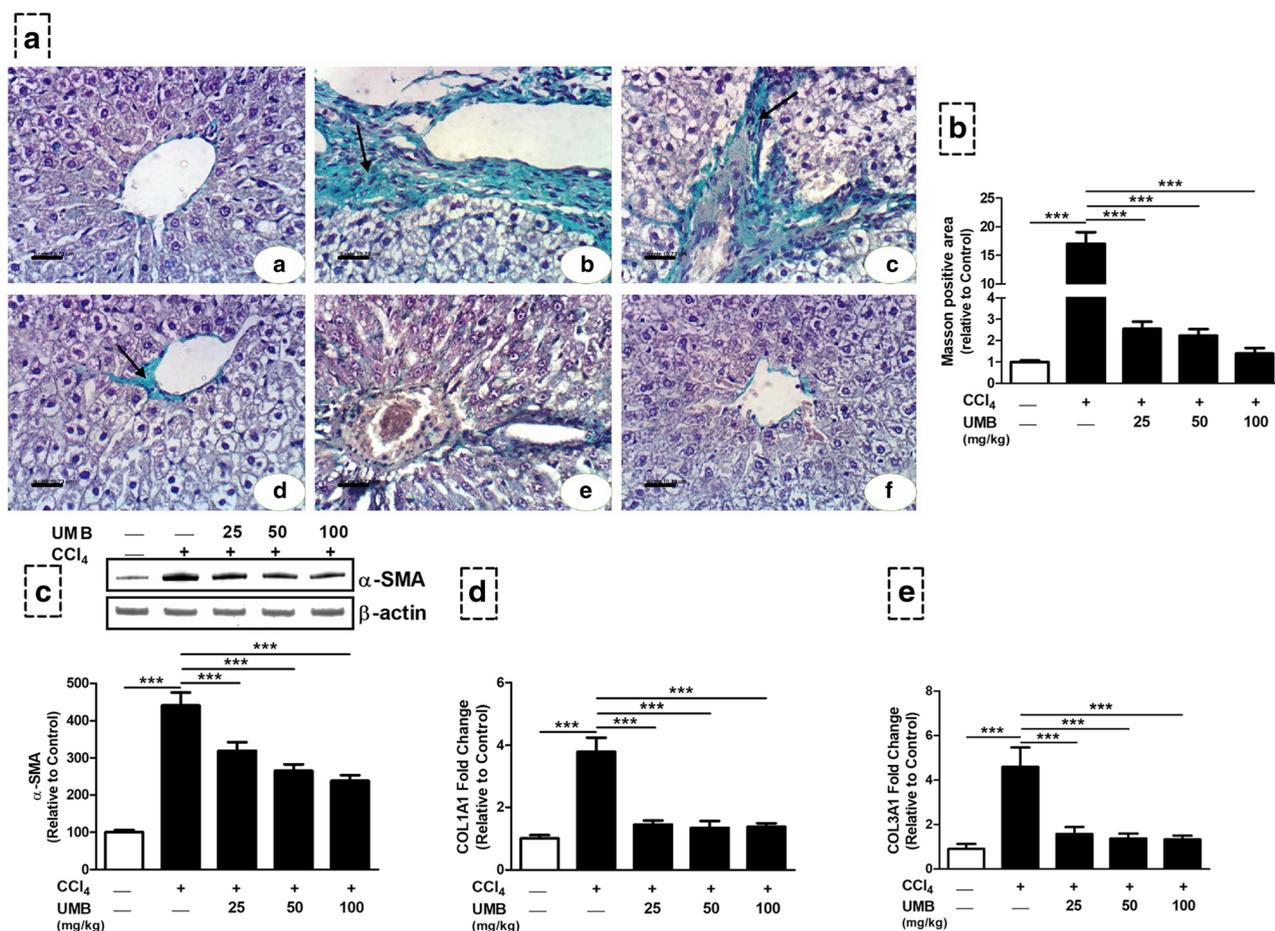
#### UMB Prevents $\text{CCl}_4$ -Induced Inflammation in Rats

To investigate the potential of UMB to attenuate  $\text{CCl}_4$ -induced inflammation in rats, we determined the expression levels of the NF- $\kappa$ B p65 subunit in the liver and circulating levels of TNF- $\alpha$  and IL-6.

$\text{CCl}_4$ -induced rats showed a notable ( $P < 0.001$ ) increase in the expression of the NF- $\kappa$ B p65 subunit in the liver. Oral supplementation of 25, 50, or 100 mg/kg UMB for 8 weeks downregulated NF- $\kappa$ B p65 in the liver of  $\text{CCl}_4$ -induced rats ( $P < 0.001$ ) as depicted in Fig. 7a.

**Table 1.** Lesion Scores of Different Liver Alterations Among All Experimental Groups

Histopathological lesions	Control	$\text{CCl}_4$	UMB (mg/kg)		
			25	50	100
Fibroblast proliferation in the capsule	–	+++	+	–	–
Fibroblast proliferation in the portal triad	–	+++	++	+	–
Focal hepatic necrosis	–	++	–	–	–
Steatosis of hepatocytes	–	+++	++	+	+
Cytoplasmic vacuolization of hepatocytes	–	+++	++	+	+
Kupffer cell activation	–	+++	+	+	+



**Fig. 4.** Umbelliferone attenuates CCl<sub>4</sub>-induced liver fibrosis in rats. **a** Masson's trichrome-stained liver sections in (a) control rats showing normal histochemical reaction for collagen fibers, (b and c) CCl<sub>4</sub>-induced rats showing strong positive histochemical reaction for collagen fibers (arrow), and CCl<sub>4</sub>-induced rats treated with 25 (d), 50 (e), and 100 mg/kg umbelliferone (f) showing slight positive histochemical reaction for collagen fibers (arrow). ( $\times 400$ ). **b** Quantification of Masson's trichrome-positive area showing a significant increase in collagen deposition and the remarkable ameliorative effect of umbelliferone. **c–e** Umbelliferone downregulated the protein expression levels of  $\alpha$ -SMA (c), and mRNA abundance of collagen I (d) and collagen III (e) in the liver of CCl<sub>4</sub>-induced rats. Data are expressed as mean  $\pm$  SEM,  $n = 6$ ,  $***P < 0.001$ .

TNF- $\alpha$  and IL-6 in serum of CCl<sub>4</sub>-induced rats showed a significant ( $P < 0.001$ ) increase when compared with those of the control group as represented in Fig. 7b, c, respectively. Treatment of the CCl<sub>4</sub>-induced rats with 25, 50, or 100 mg/kg UMB significantly ameliorated serum TNF- $\alpha$  (Fig. 7b) and IL-6 (Fig. 7c).

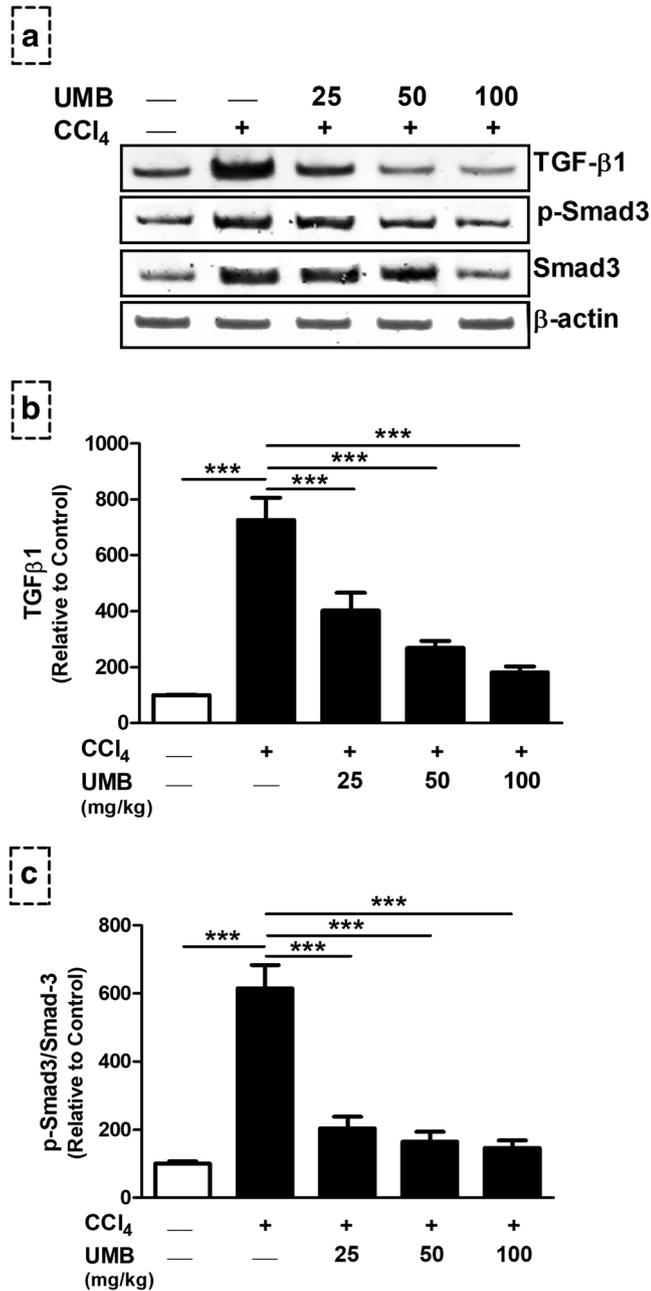
#### UMB Upregulates PPAR $\gamma$ Expression in the Liver of CCl<sub>4</sub>-Induced Rats

Several studies have demonstrated the role of PPAR $\gamma$  in the inhibition of hepatic fibrosis [13, 22, 23]. Therefore, we determined whether the antifibrosis effect of UMB was associated with PPAR $\gamma$  activation. CCl<sub>4</sub>-induced rats

showed a significant ( $P < 0.001$ ) downregulation of hepatic PPAR $\gamma$  protein expression when compared with the control rats (Fig. 8). In contrast, treatment of the CCl<sub>4</sub>-induced rats with 25, 50 or 100 mg/kg UMB resulted in a significant increase in the protein expression levels of PPAR $\gamma$ .

#### DISCUSSION

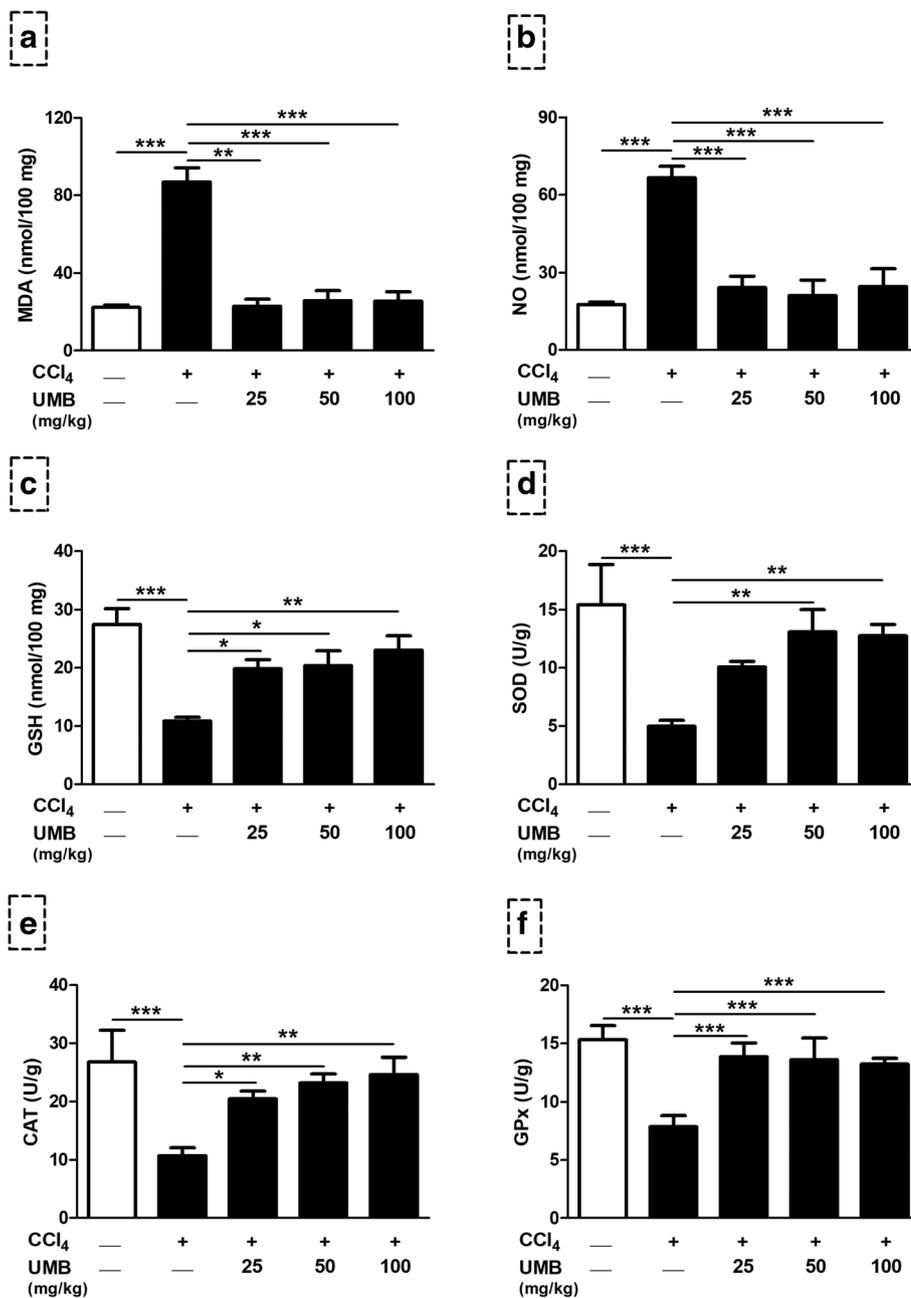
UMB has displayed several pharmacological and biological effects, including antioxidant, anti-inflammatory, and antidiabetic [24, 25]. However, its potential to protect against liver fibrosis has not been reported yet. Herein, we



**Fig. 5.** Umbelliferone downregulates TGF-β1/Smad3 signaling in the liver of CCl<sub>4</sub>-induced rats. **a** Representative blots of TGF-β1, p-Smad3, Smad3, and β-actin. Treatment with umbelliferone significantly decreased TGF-β1 protein expression (**b**) and Smad3 phosphorylation (**c**) in the liver of CCl<sub>4</sub>-induced rats. Data are expressed as mean ± SEM, *n* = 6, \*\*\**P* < 0.001.

investigated the ameliorative effect of UMB on liver fibrosis induced by CCl<sub>4</sub> in rats. Our findings introduced novel information on the hepatoprotective mechanism of UMB and pointed to the involvement of TGF-β1/Smad3

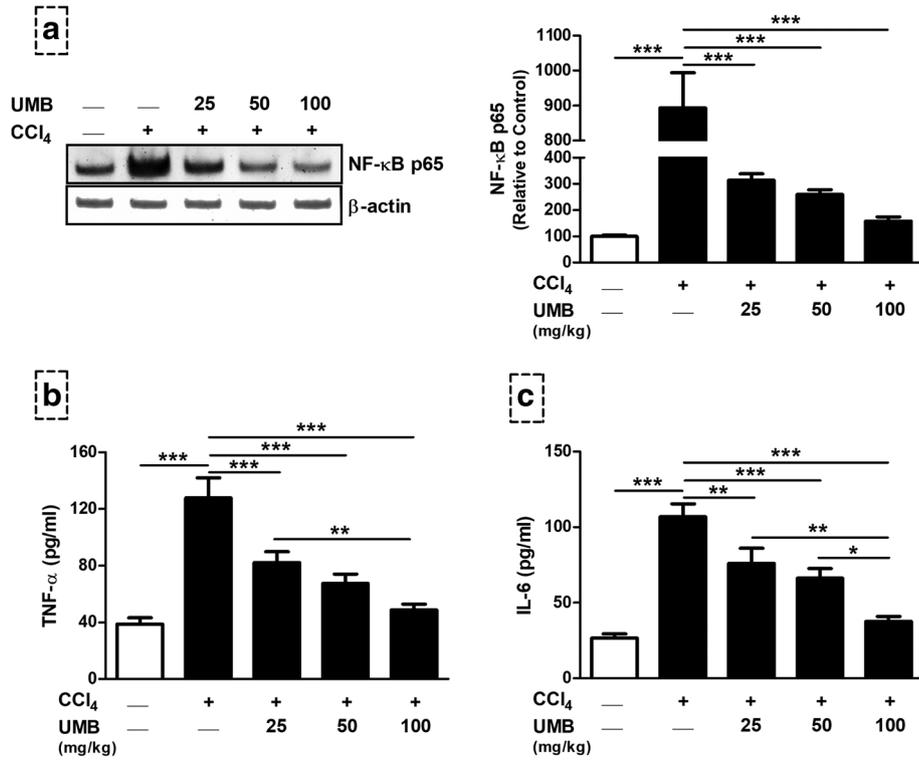
signaling and PPARγ modulation in mediating its effect. In the present study, the repeated administration of CCl<sub>4</sub> (twice per week for 8 weeks) induced hepatocellular damage and fibrosis evidenced by the increased serum ALT,



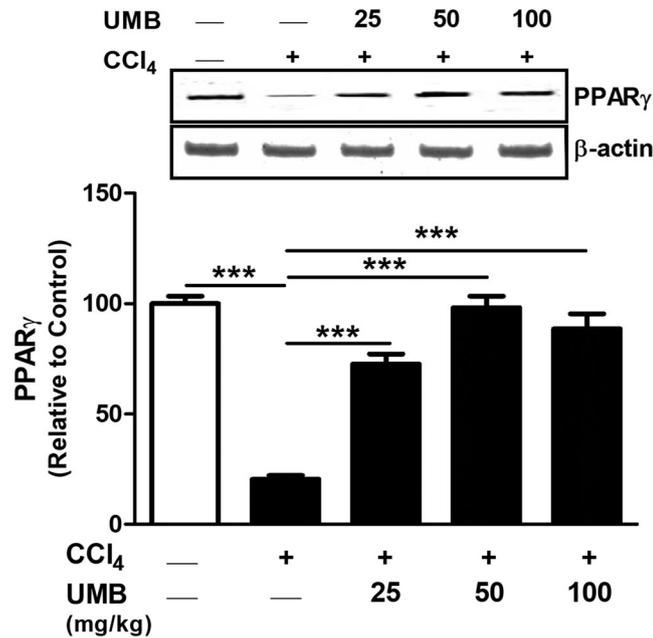
**Fig. 6.** Umbelliferone attenuates CCl<sub>4</sub>-induced oxidative stress in the liver of rats. Umbelliferone decreased MDA (a) and NO levels (b), and increased GSH (c), SOD (d), CAT (e), and GPx (f) in the liver of CCl<sub>4</sub>-induced rats. Data are expressed as mean  $\pm$  SEM,  $n = 6$ . \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

AST, ALP,  $\gamma$ GT, and bilirubin coupled with decreased serum albumin levels. Liver injury due to CCl<sub>4</sub> administration was confirmed by the histopathological manifestations, including focal hepatic necrosis, steatosis of hepatocytes, cytoplasmic vacuolization of hepatocytes, Kupffer

cell activation, and fibroblast proliferation. Staining with MT demonstrated a remarkable increase in fibrous tissue and liver fibrosis in CCl<sub>4</sub>-intoxicated rats. Liver fibrosis was further confirmed by the significant increase in the expression of  $\alpha$ -SMA, collagen I, and collagen III.



**Fig. 7.** Umbelliferone prevents CCl<sub>4</sub>-induced inflammation in rats. **a** Umbelliferone downregulated NF-κB p65 protein expression levels in the liver of CCl<sub>4</sub>-induced rats. **b** and **c** Umbelliferone decreased serum levels of TNF-α (**b**) and IL-6 (**c**) in CCl<sub>4</sub>-induced rats. Data are expressed as mean ± SEM, *n* = 6. \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001.



**Fig. 8.** Umbelliferone upregulates PPAR<sub>γ</sub> expression in the liver of CCl<sub>4</sub>-induced rats. Data are expressed as mean ± SEM, *n* = 6, \*\*\**P* < 0.001.

Interestingly, all alterations induced by the chronic administration of CCl<sub>4</sub> were significantly attenuated by UMB. Treatment with different doses of UMB remarkably ameliorated the liver function markers and prevented histological alterations induced by CCl<sub>4</sub>. In addition, UMB inhibited fibroblast proliferation and suppressed the production and deposition of fibrous ECM as evidenced by downregulation of  $\alpha$ -SMA and collagen expression. Collagen I and collagen III, the main components of ECM, are synthesized by fibroblasts, and their excessive secretion and accumulation represent the characteristic pathological feature in liver fibrosis [5]. Excessive ECM deposition along with limited remodeling occur during chronic liver disease and lead to scar deposition and fibrosis marked by excessive deposition of ECM extensively rich in both collagen I and collagen III [41]. Recent studies have demonstrated increased hepatic collagen and  $\alpha$ -SMA expression in CCl<sub>4</sub>-intoxicated rodents [6, 32]. Therefore, UMB reversed the progression of fibrosis by suppressing the excessive deposition of ECM *via* downregulating collagen I, collagen III, and  $\alpha$ -SMA.

To explore the mechanism underlying the ameliorative efficacy of UMB on CCl<sub>4</sub>-induced liver injury and fibrosis, we determined its modulatory effect on TGF- $\beta$ 1/Smad3 signaling. Previous studies have demonstrated the central role of TGF- $\beta$ 1 in liver fibrosis by promoting myofibroblast-like cell formation and inducing the excessive accumulation of ECM components. TGF- $\beta$ 1 binds to its transmembrane receptor and activates Smad signaling, leading to increased expression of the ECM proteins [8]. Herein, CCl<sub>4</sub>-intoxicated rats showed a significant increase in liver TGF- $\beta$ 1 expression and Smad3 phosphorylation levels. Accordingly, previous studies have reported increased TGF- $\beta$ 1 and Smad3 expression in CCl<sub>4</sub>-induced rats [6, 32]. In a rat model of CCl<sub>4</sub>/diethylnitrosamine (DEN)-induced hepatocarcinogenesis, we have recently shown that increased deposition of ECM and liver fibrosis were associated with TGF- $\beta$ 1/Smad3 signaling activation [14]. Here, treatment with UMB decreased the protein expression and phosphorylation levels of TGF- $\beta$ 1 and Smad3, respectively, in the liver of CCl<sub>4</sub>-intoxicated rats. Therefore, suppressed TGF- $\beta$ 1/Smad3 signaling is the main mechanism by which UMB reversed the progression of fibrosis in CCl<sub>4</sub>-intoxicated rats. This notion has been supported by the study of Lang et al. who investigated the role of TGF- $\beta$ 1 silencing in the attenuation of fibrosis [42]. siRNA-mediated downregulation of TGF- $\beta$ 1 inhibited the proliferation of HSCs and suppressed the production of collagen I and collagen III, leading to prevention of liver fibrosis in rats [42].

Oxidative stress plays a critical role in liver fibrogenesis as revealed by several studies [6, 14, 32]. Increased ROS production has been associated with the activation of HSCs and production of collagen-rich ECM [6, 7]. In addition, oxidative stress-mediated fibrosis occurs due to excessive ROS production and its subsequent hepatocyte injury *via* membrane lipid peroxidation [43]. CCl<sub>4</sub>-derived free radicals cause hepatocyte membrane damage and leakage of the enzymes into the circulation [44], as confirmed by the elevated serum levels of ALT, AST, ALP, and  $\gamma$ GT. Within the liver, CCl<sub>4</sub> is metabolized into the highly reactive trichloromethyl and peroxy radicals, *via* the action of cytochrome P450. These radicals initiate lipid peroxidation and can bind covalently to the cellular macromolecules [44]. In addition, CCl<sub>4</sub> has been recently reported to induce nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX)-mediated ROS production in the liver of rats [6]. In our study, oxidative stress was monitored by measuring MDA, NO, GSH, and antioxidant enzymes. MDA, a marker of lipid peroxidation, and NO levels were significantly increased in CCl<sub>4</sub>-intoxicated rats. Lipid peroxidation is mediated *via* CCl<sub>4</sub>-derived radicals and increased ROS generation, whereas increased NO could be attributed to increased expression of inducible nitric oxide synthase (iNOS). NF- $\kappa$ B is a transcription factor very well known to be activated by ROS. Activated NF- $\kappa$ B promotes the expression of several inflammatory mediators, including TNF- $\alpha$ , IL-6, and iNOS [16]. Here, CCl<sub>4</sub> increased the expression of liver NF- $\kappa$ B p65 and serum levels of TNF- $\alpha$  and IL-6. Chronic inflammation has been reported to precede fibrosis and to be associated with the development of cirrhosis [45]. Apoptotic bodies derived from damaged hepatocytes due to inflammation can activate Kupffer cells and HSCs, promoting inflammation and fibrogenesis in the liver [5]. Histological examination of the liver of CCl<sub>4</sub>-induced rats revealed activated Kupffer cells associated with fibroblast proliferation. Activated Kupffer cells can stimulate HSCs through the release of cytokines and chemokines [5, 46]. In addition, ROS and NO produced by the active Kupffer cells can stimulate DNA damage, apoptosis, and expression of pro-inflammatory genes, leading to fibrogenesis [47]. In conjunction with increased MDA and NO, CCl<sub>4</sub>-induced rats exhibited a decrease in liver GSH content and the activity of SOD, CAT, and GPx. GSH is an essential antioxidant that scavenges free radicals and maintains protein sulfhydryl groups, and SOD, CAT, and GPx are antioxidant enzymes that protect the cells against the deleterious effects of free radicals and oxidants. Therefore, depletion of these antioxidant defenses can lead to cell injury and death. In accordance with our findings, recent

studies have demonstrated increased lipid peroxidation and diminished GSH and SOD in the liver of CCl<sub>4</sub>-intoxicated rats [6, 32]. Treatment of the CCl<sub>4</sub>-intoxicated rats with UMB significantly decreased MDA and NO and boosted the antioxidant defenses. In addition, UMB suppressed inflammation in CCl<sub>4</sub>-intoxicated rats as revealed by the downregulated NF- $\kappa$ B and decreased TNF- $\alpha$  and IL-6. We have previously reported the efficacy of UMB to prevent cyclophosphamide hepatotoxicity *via* suppression of lipid peroxidation, NF- $\kappa$ B and iNOS, and enhancement of the antioxidant defense system [17]. Therefore, attenuation of oxidative stress and inflammation plays a central role in the ameliorative effect of UMB against liver fibrogenesis.

The crucial role of oxidative stress and inflammation in chronic liver disease and the deleterious effects of ROS and pro-inflammatory cytokines on hepatocytes support the use of UMB as an antifibrotic agent. The antioxidant, anti-inflammatory, and antifibrotic potential of UMB could be explained by its ability to activate the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and PPAR $\gamma$  signaling as we previously reported [17]. In support of our findings, Yin et al. have recently shown the ability of UMB to upregulate Nrf2 in the liver of diabetic mice [27]. When activated, the redox-sensitive Nrf2 can attenuate oxidative stress and suppress NF- $\kappa$ B, iNOS, and pro-inflammatory cytokines [16, 19, 48]. The ligand-activated nuclear receptor PPAR $\gamma$  possesses potent anti-inflammatory properties mediated *via* negative interference and/or transcriptional repression of NF- $\kappa$ B [11], and can directly induce expression of the antioxidant enzymes [12]. The role of PPAR $\gamma$  in attenuating the progression of liver fibrosis has been reported in different studies. Yang et al. have reported enhanced fibrogenesis in PPAR $\gamma$ -deficient mice after liver injury, whereas HSC activation and fibrosis were suppressed following *in vivo* PPAR $\gamma$  overexpression [22]. In CCl<sub>4</sub>-induced fibrosis in mice, PPAR $\gamma$  suppressed the differentiation of bone marrow-derived mesenchymal stem cells into myofibroblasts [49]. PPAR $\gamma$  can also interact with the fibrosis-related signaling pathway TGF- $\beta$ 1/Smad3. PPAR $\gamma$  has been reported to bind Smad3 and prevent the nuclear accumulation of p-Smad3, leading to suppression of TGF- $\beta$ 1 signaling [13]. We have previously reported a negative correlation between PPAR $\gamma$  upregulation and TGF- $\beta$ 1/Smad3 pathway in the liver of CCl<sub>4</sub>/DEN-induced rats [14]. The PPAR $\gamma$  agonist rosiglitazone exhibited antifibrotic function mediated *via* suppressing the early growth response protein 1 (Egr-1) which acts as a mediator of non-Smad TGF- $\beta$ 1 signaling [50]. The antifibrotic potential of UMB is therefore associated with its ability to upregulate PPAR $\gamma$  and subsequently attenuate

inflammation and fibrosis. However, the role of PPAR $\gamma$  in mediating the antifibrotic mechanism of UMB needs to be further explored.

In conclusion, our results demonstrated that UMB prevented CCl<sub>4</sub>-induced liver fibrosis in rats. UMB attenuated oxidative stress, inflammation, expression of collagen and  $\alpha$ -SMA, and deposition of ECM in the liver of rats. In addition, UMB suppressed TGF- $\beta$ 1/Smad3 signaling and upregulated the expression of PPAR $\gamma$ . Therefore, UMB may be a promising candidate for preventing the progression of fibrogenesis. However, further research is needed to delineate the exact molecular mechanisms underlying the antifibrotic efficacy of UMB.

## AUTHORS' CONTRIBUTION

AMM, WGH, and IHH conceived the study and designed the experiments. AMM and ES performed the experiments. AMM analyzed the data, prepared the figures, and wrote the manuscript. All the authors participated in the assays, revised the manuscript, and approved the submission.

## COMPLIANCE WITH ETHICAL STANDARDS

The experimental protocol and all animal procedures were approved by the Institutional Animal Ethics Committee of Beni-Suef University (Egypt).

**Conflict of Interest.** The authors declare that they have no conflict of interest.

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