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Omama E. El-Shawia, Heba A. S. El-Nasharb,c, Sahar S. Abd El-Rahmand, Omayma A. Eldahshanb,c, and Abdel Nasser B. Singab,c

*Health Radiation Research Department, National Centre for Radiation Research and Technology, Atomic Energy Authority, Cairo, Egypt;"*Department of Pharmacognosy, Faculty of Pharmacy, Ain Shams University, Abbassia, Cairo, Egypt;"*Center for Drug Discovery Research and Development, Ain Shams University, Egypt;"*Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt

**ABSTRACT**

**Purpose:** Liver fibrosis is considered as one of the ultimate outcomes of chronic liver disorders, characterized by outrageous cell proliferation and abnormal deposition of extracellular matrix, resulting in severe pathological distortions in the architecture and performance of liver tissues. The present study aimed to investigate the protective properties of aqueous methanol extract of *Acrocarpus fraxinifolius* leaves (AFL) against liver fibrosis induced by dual toxicity of γ-irradiation and carbon tetrachloride (CCl₄) in rats.

**Methods:** The animals were exposed to 2 Gy irradiation once/week concurrently with intraperitoneal administration of CCl₄ (0.2 mL/100 g body weight) for seven weeks. Afterwards, liver toxicity and fibrosis were assessed biochemically at cellular and molecular as well as histopathological levels.

**Results:** The livers of intoxicated rats showed distinct structural and functional changes, compared with the normal rats. The administration of AFL (500 mg/kg, *p.o.*) significantly ameliorated the histopathological manifestations of fibrotic liver evidenced by mitigated steatosis progression, necrosis, fibrotic septa, apoptotic bodies, and immunohistochemical studies of alpha-smooth muscle actin. Also, AFL increased the final body weight, total protein, albumin levels and albumin/globulin ratio. While, the absolute liver weight, liver enzymes, total cholesterol and triglycerides were reduced. A significant modulation was observed in hydroxyproline, transforming growth factor-β and collagen-1 expression. Furthermore, AFL exerted a direct effect on liver fibrosis by promoting extracellular matrix degradation via overexpression of the tissue inhibitor metalloproteinase-1, coupled with decrease of metalloproteinase-9 activity.

**Conclusions:** Our findings suggested that AFL effectively improved the architecture of fibrotic liver and modified the biochemical markers of liver fibrosis.

**GRAPHICAL ABSTRACT**

**ARTICLE HISTORY**

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Liver; fibrosis; carbon tetrachloride; radiation; *Acrocarpus fraxinifolius*; metalloproteinase

**CONTACT**

Abdel Nasser B. Singab  
Dean@pharma.asu.edu.eg; Omayma A. Eldahshan  
oeldahshan@pharma.asu.edu.eg  
Department of Pharmacognosy, Faculty of Pharmacy, Ain Shams University, Cairo 11566, Egypt

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Introduction

Liver fibrosis is a pathological condition marked by an outrageous accumulation and deposition of extracellular matrix (ECM) proteins in hepatic tissues during healing process of chronic liver diseases (Bataller and Brenner 2005). A disequilibrium between ECM overproduction and decomposition resulted in the eventual development of liver fibrosis (Wang et al. 2018). These complex alterations induce distortion and disruption of tissue structure and functions in the liver throughout liver fibrosis (Baues et al. 2017). Liver fibrosis elicits significant morbidity and mortality that affects about 100 million people worldwide (Sánchez-Valle et al. 2012). Egypt has the highest prevalence of HCV infection in the world, affecting about 15%–25% of the population in rural communities (Frank et al. 2000).

Carbon tetrachloride (CCl₄) is a laboratory hepatotoxic agent, that is widely used to provoke experimental liver lesion and hepatic fibrosis (Dong et al. 2016). The metabolism of CCl₄ is induced by liver cytochrome P450 resulting in production of highly reactive trichloromethyl radicals (CCl₃⁺) and trichloromethyl peroxy radicals (CCl₃O₂⁻) (El-Nashar et al. 2021a). Consequently, these reactive free radicals (ROS) initiate lipid peroxidation and decrease the membrane permeability of all cellular compartments which cause hepatic damage, fibrosis, cirrhosis and sometimes hepatocellular carcinoma (Scholten et al. 2015).

During treatment of cancer patients by radiation therapy, the patients are usually exposed to highly fractionated dose of γ-radiation (2 Gy/day) especially of the upper abdomen or whole abdomen regions (Kim and Jung 2017). Radiation therapy has played a minor role in the treatment of patients with liver cancer or liver metastases because the liver has been considered sensitive to radiation (Malik et al. 2010). Single-dose γ-irradiation of rat liver changes the gene expression of several proteins including those of iron metabolism. Additionally, up-regulation of the genes of some proinflammatory chemokines (CINC-1/CXCL8, IP-10/CXCL10, ITAC/CXCL11, MCP-1/CCL2, MIG/CXCL9, MIP-1b/CCL3, MIP-3b/CCL20, MMP-2 and MMP-9 activate pro-TGF-β which is rapidly released, promoting collagen synthesis and fibrosis (Reggio et al. 2013).

Collagen embodies the substantive building block of ECM protein in mammalian tissues and constitutes about 30% of the total body protein (Arriazu et al. 2014). The collagen degradation is essentially involved in the tissue development, morphogenesis, remodeling and repair; it is tightly regulated in the normal physiological conditions, but its dysregulation leads to many disorders including fibrosis (Jablonska-Trypuć et al. 2016). Thus, the deposition of collagen amplifies the fibrotic ECM deposition (Schuppan et al. 2001).

Because of the reversibility of liver fibrosis in the initial steps, the effective treatment is clinically very crucial as well therapeutic strategies are highly demanded in this field to avoid the progress of hepatic fibrosis toward dangerous cases of cirrhosis and hepatic cancer (Li et al. 2019). Various plant extracts and natural products have been referred for treatment of wide spectrum of liver diseases (Ghoneim and Eldahshan 2012; Azab et al. 2013; Mostafa et al. 2018; El-Nashar et al. 2020b). Acrocarpus fraxinifolius is one of leguminous trees belonging to caesalpinioideae subfamily-caesalpiniae tribe (El-Nashar et al. 2017). Previously, we carried out a phytochemical study on the leaf extract of Acrocarpus fraxinifolius and identified phenolic constituents including tannins, flavonoids, and glycosides. This extract elicited a significant hepatoprotective effect via antioxidant, anti-inflammatory, and anti-apoptotic activity in rats (El-Nashar et al. 2017).

The present study aimed to evaluate the effect of Acrocarpus fraxinifolius (AFL) extract on γ-irradiation and CCl₄ experimentally-induced liver fibrosis in rats, with point stellate cells (HSC), which is an important event in the cascade of liver fibrosis and commonly regarded as major producers of fibrotic ECM proteins (Ramos-Tovar and Muriel 2020). Transforming growth factor-β (TGF-β) signaling plays an essential role in the initial liver injury, inflammation, fibrosis and cirrhosis (Caja et al. 2018). Additionally, TGF-β is produced by Kupffer cells or damaged hepatocytes and binds to a heteromeric TGF-β receptor complex consisting of type I and type II serine/threonine receptors, which further cause phosphorylation of Smad2 and Smad3, and subsequent binding to Smad4 then translocation from the cytosol into nucleus, followed by stimulation of pro-fibrotic mediators and transcription of collagen I, α-smooth muscle actin (α-SMA) and tissue inhibitors of matrix metalloproteinases (TIMPs) expression, thereby accumulation and deposition of extracellular matrix occurs (Hung et al. 2017).

There are highly specialized proteolytic enzymes, called metalloproteinases (MMPs), implicated in the degradation of ECM components and progression of fibrotic liver disorders (Bourboula and Settler-Stevenson 2010). There is a balance between TIMPs and MMPs in healthy liver, which control the deposition and removal of the ECM. During fibrogenesis, this equilibrium is disturbed with overexpression of TIMPs/MMPs ratio and thus matrix degradation is inhibited (Roeb 2018). In case of ECM remodeling, MMPs such as MMP-2 and MMP-9 activate pro-TGF-β which is rapidly released, promoting collagen synthesis and fibrosis (Reggio et al. 2013).
of view to understand the possible underpinning mechanisms.

**Materials and methods**

**Plant material and extraction**

The fresh leaves of *Acrocarpus fraxinifolius* (Fabaceae) were identified, authenticated, and collected from Egyptian Botanical Zoo Garden, Giza, Egypt. A Voucher specimen of the leaves is deposited with code of PHG-P-AF130 at herbarium of Pharmacognosy Department, Faculty of Pharmacy, Ain Shams University. The aqueous methanol extract of *Acrocarpus fraxinifolius* leaves (AFL) was prepared according to the method described by El-Nashar et al. (2017).

**Chemicals and kits**

Carbon tetrachloride (CCl₄) was brought from Sigma-Aldrich, Germany. The biochemical analysis kits of liver markers, cholesterol and triglycerides were purchased from Bio-diagnostic Company, Egypt. The kits for biochemical analysis of total protein, albumin and globulin were brought from spinreac kits, Spain. ELISA Immuno-assay (Procine/Canine) kit with Catalog Number MB100B was supplied by R&D Quantikine, USA.

**Selection of experimental animals, caring and handling**

Male albino rats weighing 110–120 g were purchased from the Egyptian Organization for Biological Products and Vaccines. The rats were acclimatized in the laboratory for one week before the commencement of the experiment. Plastic cages with wire mesh covers were used to keep rats under normal environmental conditions of temperature, humidity and 12-hour light/dark cycle. A commercial standard diet and water *ad libitum* were continuously and regularly supplied throughout the whole experimental period. We followed the guidelines of World Health Organization at Geneva, Switzerland during animal handling and care. Also, the animal care committee of the National Center for Radiation Research and Technology (NCRRT) hosted the experiment and approved handling issues, Atomic Energy Authority (AEA), Cairo, Egypt.

**Irradiation facility**

The source of γ-radiation was provided by A Canadian Gamma Cell-40 (¹³⁷Cs) at the National Center of Radiation Research and Technology (NCRRT), Cairo, Egypt. The dose rate was 0.657 Gy/minute as calibrated during the study period. For concessive seven weeks, the animals were exposed to 2 Gy per week up to a total dose of 14 Gy of irradiation intoxication.

**Induction of liver fibrosis**

A dual toxicity of γ-irradiation and CCl₄ experimentally induced liver fibrosis in male albino rats. Rats exposed to repeated doses of γ-irradiation for seven weeks (2 Gy once/week up to total dose of 14 Gy), with concurrent intraperitoneal injection of CCl₄ in olive oil (1:1, V/V) at a dose of 0.2 mL/100g body weight twice/week.

**Study design and experimental protocol**

A selection of forty male albino rats weighing about 110–120gm were divided into four groups; control group (n = 5) received (i.p.) 0.2 mL/100g body weight of olive oil in saline solution (V/V), AFL extract group (n = 5) animals received 1 mL of AFL extract (500 mg/100 g body weight) dissolved in saline solution orally twice/week, CCl₄ group were given (i.p.) 0.2 mL of 2 mL/kg b.w of CCl₄ (dissolved in olive oil 1:1 volume) twice a week (Monday and Wednesday), IRR + CCl₄ group (n = 15) received 2 Gy γ-irradiation followed by 0.2 mL/100g body weight of CCl₄ and olive oil (i.p). The experimental group (IRR + CCl₄+AFL, n = 15) received 5 doses of AFL (500 mg/100 g body weight) one week before the dual toxicity. Thereafter, the experimental group received 500 mg/100g body weight twice weekly simultaneously with the dual toxicity for seven weeks.

**Samples collection**

After experimentation period finished, the rats were weighed, and anesthetized by diethyl ether. Heparinized test tubes were used for collection of blood samples, and allowed to stand for 30 min for clotting, then the plasma was separated by centrifugation at 3000 rpm at 4°C. Liver samples were isolated, grossly examined, then, a portion of the liver of each animal was immediately taken out and washed with ice-cold saline, stored at −20°C and processed for biochemical analysis.

**Histopathological studies**

Freshly prepared neutral buffered formalin (10%) was used to fix liver samples, then they were dehydrated by graded series of alcohol and put in paraffin (Bancroft and Gamble 2008). Then, the liver sections were prepared serially at 4–5 μm thickness and finally stained by hematoxylin and eosin.

**Immunohistochemical examination**

Immunohistochemical studies were performed to detect alpha-SMA expression of liver sections using avidin-biotin peroxidase. The liver sections were incubated with a monoclonal antibody (Dako Corp, Carpinteria, CA), then the marker was visualized by 3,3-diamino benzidine tetrahydrochloride (DAB, Sigma).
Biochemical analysis of the plasma

Plasma parameters such as ALT and AST activities were assessed according the method described by (Reitman and Frankel 1957). Also, the total protein (TP), albumin (A), globulin (G) and A/G ratio according the method described by Doumas et al. (1971). The total lipids (TP), total cholesterol (TC) and triglyceride (TG) levels were analyzed according to reported methods of (Richmond 1973; Fossati and Prencipe 1982).

Biochemical analysis of the hepatic tissue

The severity of liver fibrosis was evaluated via measurement of hydroxyproline (HP) and TGF-β levels (Nakamura et al. 2000). Also, the expression levels of TGF-β1 and collagen type-1 were quantified using real time-polymerase chain reaction (RT-PCR) technique. Further, western blot immunoassay detected the expression of matrix metalloproteinase (MMP-9) and tissue inhibitor metallopeptidase inhibitor-1 (TIMP-1) enzymes in the hepatic tissue.

RNA isolation and RT-PCR analysis

One-step Qiagen RT-PCR kit was used to assess the effects of AFL extract on hepatic mRNA of TGF-β1 and collagen type-1 from each group (Livak and Schmittgen 2001). The extraction and purification of hepatic RNA were done according to RNeasy kit protocols (Qiagen, Valencia, California, USA). The glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as an internal control agent; the primer pairs are listed in Table 1.

Western blot immunoassay

The effect of AFL extract on MMP-9 and TIMP-1 protein levels in the different treated groups was examined using western blotting immunoassay. Samples with equivalent amounts of 5µg of total protein were loaded to SDS-PAGE gel, then transferred to PDVF membrane by electrophoresis. A dry milk (5% non-fatty) was used to block the blots in TBST at room temperature for 3 hr. After blocking, blots were probed with specific primary antibodies ‘Mouse monoclonal primary antibodies [56-2A4 (ab 58803) and 2E7.1 (ab28261)] 114 specific for MMP-9 and TIMP-1, respectively’. The membranes were then washed 3 times with 0.1% Tween and TBST, then incubated with anti-mouse and horseradish peroxidase labeled IgG (cell signaling, USA) as secondary antibodies (1:3000) dilution at room temperature for 1 hr. the protein bands were then detected by chemiluminescence reagent (Pierce ECL Western blotting substrate) and Alliance Gel-doc. The band intensity was analyzed by Alliance 4.7 Gel-doc, UVtec software, UK. All blots were normalized against intensities of corresponding β-actin protein bands.

Statistical analysis

One-way analysis of variance (ANOVA) was utilized to analyze the statistical differences between the studied groups. All values of results are represented as mean ± standard error of mean (SEM). A significant data is expressed by p value <.05. The data analysis was implemented by SPSS 20 software.

Results

Histopathological studies

As shown in Figure 1, the livers of the control (Figure 1(A)) and AFL extract group (Figure 1(B)) appeared smooth shiny surface, while the CCl4 group (Figure 1(C)) and IRR + CCl4 group (Figure 1(D)) showed marked distortion of the liver structure with pale-color, rough, hard, fatty, patchy surface of liver. In addition, the fibrotic livers of IRR + CCl4 injured rats (Figure 1(D)) showed nodular appearance and faint red color indicating a decreased in blood amount. Whereas administration of AFL extract (500 mg/kg) restored the normal architecture of liver of IRR + CCl4+AFL (Figure 1(E)); the liver appeared shinier, and smoother compared with that of the fibrotic model. Microscopically, the control and AFL groups revealed normal histological structure of hepatic parenchymal.

Table 1. Primer sequences used for TGF-B and collagen-1 RT-PCR analysis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
</tr>
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<tbody>
<tr>
<td>TGF-β1</td>
<td>Forward primer: 5'-AGGGCTACCATGCCACCTCTC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5'-GGCGACGGCATACGACGGAT-3'</td>
</tr>
<tr>
<td>Collagen-1</td>
<td>Forward primer: 5'-AACTTGAACGTGTAACGGC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5'-CACCGATAGGGCGGTTGC-3'</td>
</tr>
<tr>
<td>GADPH</td>
<td>Forward primer: 5'-CTGCCCACTCTCCTCCACATTGTGC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5'-CTTGCTCTACGATCATCCTGGC-3'</td>
</tr>
</tbody>
</table>

Figure 1. Gross appearance of liver in different treated group showed (A) Control group, (B) AFL extract, (C) CCl4 group, (D) fibrosis model rats (dual toxins gamma-irradiation and CCl4), and (E) experimental group (AFL extract and dual toxins).
cells, vasculature and portal triads of liver tissue as shown in Figures 2(A and B). While the examination of liver sections of CCl₄ and IRR + CCl₄ groups revealed massive alteration in the normal hepatic histology. The CCl₄ group showed positive blue colored collagen fibers in the fibrous septa with pseudolobulation of the hepatic parenchyma (Azan stain × 200). (D) Liver of CCl₄ group showing distortion of the hepatic plate with remarkable hepatocytes vacuolar degeneration and fat steatosis (H&E X100). (E) The liver sections of IRR + CCl₄ group revealed massive destruction and alteration in the normal hepatic histology. (F) The liver sections of IRR + CCl₄ group with complete distortion of the arrangement of the hepatic plate, marked hepatocellular vacuolar degeneration and fat steatosis (arrow), occasional formation of fat cyst (dashed arrow) and hepatic cells necrosis in fragments (arrow) with increased number of apoptotic bodies, (G) Bridging fibrosis notice the neighboring portal areas are bridged by fibrous septa (dashed arrow) with the formation of marked pseudolobules (PS) (H) (H&E, X200 and 400). (I) Liver of AFL + IRR + CCl₄ group showing limitation of collagen proliferation to the portal area, decreased number of apoptotic bodies (arrow) and no obvious pseudolobulation (H&E × 400). (J) Liver of AFL + IRR + CCl₄ group showing improvement of the state of steatosis and collagen proliferation in the portal area.

Figure 2. The effect of AFL extract on the Pathological features of rat liver tissue in different groups. (A) Livers of control, (B) rats delivered AFL extract only showed normal histological structure. (C) Liver of CCl₄ group with positive blue colored collagen fibers in the fibrous septa with pseudolobulation of the hepatic parenchyma (Azan stain × 200). (D) Liver of CCl₄ group showing distortion of the hepatic plate with remarkable hepatocytes vacuolar degeneration and fat steatosis (H&E X100). (E) The liver sections of IRR + CCl₄ group revealed massive destruction and alteration in the normal hepatic histology. (F) The liver sections of IRR + CCl₄ group with complete distortion of the arrangement of the hepatic plate, marked hepatocellular vacuolar degeneration and fat steatosis (arrow), occasional formation of fat cyst (dashed arrow) and hepatic cells necrosis in fragments (arrow) with increased number of apoptotic bodies, (G) Bridging fibrosis notice the neighboring portal areas are bridged by fibrous septa (dashed arrow) with the formation of marked pseudolobules (PS) (H) (H&E, X200 and 400). (I) Liver of AFL + IRR + CCl₄ group showing limitation of collagen proliferation to the portal area, decreased number of apoptotic bodies (arrow) and no obvious pseudolobulation (H&E × 400). (J) Liver of AFL + IRR + CCl₄ group showing improvement of the state of steatosis and collagen proliferation in the portal area.
apparently suppressed hepatic fibrosis with its limitation to the portal areas as well as reducing the thickness of incomplete fibrotic septa with no obvious pseudolobulation. The immunohistochemistry examination of livers of control and AFL groups (Figure 3(A–C)) revealed normal positive expression of α-SMA around central veins and portal veins with normal myofibroblasts, while livers of CCl4 and IRR + CCl4 groups revealed a marked increased immunopositivity of α-SMA in the portal triads along with proliferated fibrous connective tissue and the bridging fibrous stands (Figure 3(D & E)). Administration of AFL extract decreased α-SMA expression appeared as scattered mild immunopositivity in the portal area and parenchyma of IRR + CCl4 + AFL group (Figure 3(F)).

**Mortalities and survival after different treatments along the experimentation**

The mortality was investigated in all studied groups along experimentation (Table 2). No mortality was detected in the control and AFL extract groups. Meanwhile, the CCl4 alone and IRR + CCl4 groups showed approximately 40% and 53% mortality, respectively. The oral treatment of AFL extract reduced the mortality to be 25% in the IRR + CCl4 + AFL group.

**Effects of AFL extract on the body weight and absolute liver**

The final body weight and absolute weight of liver in AFL extract alone did not show any significant difference (p > .05). Whereas CCl4 alone group and the dual toxicity of IRR + CCl4 group caused significant decrease (p < .001) in the final body weight with marked increase in liver weight (p < .001), compared with the control group. The oral administration of AFL extract (500 mg/100g b.w) simultaneously with the dual toxins exerted a profound protective effect in IRR + CCl4 + AFL group as manifested by the significant increase of the final body weight (p < .01) and significant decrement of the absolute liver weight (p < .001), compared with the fibrotic model (Table 3).

**Biochemical analysis of the plasma**

**Effects of AFL extract on the body weight and absolute liver**

The final body weight and absolute weight of liver in AFL extract alone did not show any significant difference (p > .05). Whereas CCl4 alone group and the dual toxicity of
IRR + CCl₄ group caused significant decrease \( (p < 0.001) \) in the final body weight with marked increase in liver weight \( (p < .001) \), compared with the control group. The oral administration of AFL extract \( (500 \text{mg/100g b.w}) \) simultaneously with the dual toxins exerted a profound protective effect in IRR + CCl₄ + AFL group as manifested by the significant increase of the final body weight \( (p < .01) \) and significant decrement of the absolute liver weight \( (p < .001) \), compared with the fibrotic model (Table 3).

**Biochemical analysis of the plasma**

A significant elevation of liver enzyme markers (ALT and AST) was observed \( (p < .0001) \) in CCl₄ alone and IRR + CCl₄ groups, compared with control group. While the concurrent administration of AFL extract \( (500 \text{mg/100g b.w}) \) in IRR + CCl₄ + AFL group markedly reduced the elevation of the enzyme activities \( (p < .001) \), compared to IRR + CCl₄ model (Figure 4).

No significant change \( (p < .05) \) was elicited in the levels of total protein (TP), albumin (A) and globulin (G) or A/G ratio in animals treated with AFL extract alone. Meanwhile, CCl₄ alone and IRR + CCl₄ groups demonstrated a significantly decline of these parameters \( (p < .001) \), compared with those of control. As illustrated in Table 4, AFL extract markedly elevated these parameters in the IRR + CCl₄ + AFL group, compared with IRR + CCl₄ model.

The AFL extract group did not significantly affect \( (p > .05) \) the total cholesterol (TC) and triglyceride (TG) levels. On the other hand, a significant elevated level \( (p < .001) \) was shown in CCl₄ alone and IRR + CCl₄ model, compared to control. Conversely, the oral treatment with AFL extract significantly restored \( (p < .001) \) them back near the normalcy in IRR + CCl₄ + AFL group as shown in Figure 5.

The effects of AFL extract on hydroxyproline (HP) and TGF-β levels of different groups are presented in Figure 6(A). On its own, AFL extract only did not elicit any marked changes \( (p > .05) \), while a significant elevation of both parameters \( (p < .001) \) was observed in the CCl₄ and IRR + CCl₄ groups, compared to that of control. Interestingly, the oral treatment with AFL extract significantly reduced HP content \( (p < .01) \) in liver tissue, reflecting decreased total hepatic collagen content. Likewise, TGF-β level was diminished \( (p < .001) \)

### Table 3. The effect of AFL extract on the body weight and absolute liver weight in different studied groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (g)</th>
<th>Absolute liver weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>219 ± 4.60</td>
<td>5.70 ± 0.28</td>
</tr>
<tr>
<td>AFL extract</td>
<td>224 ± 6.20</td>
<td>6.1 ± 0.17</td>
</tr>
<tr>
<td>CCl₄</td>
<td>179 ± 7.20</td>
<td>8.9 ± 0.16 ( ^{0.14%} )</td>
</tr>
<tr>
<td>IRR + CCl₄</td>
<td>168.20 ± 4.30</td>
<td>9.05 ± 0.59 ( ^{8.70%} )</td>
</tr>
<tr>
<td>IRR + CCl₄ + AFL</td>
<td>207.80 ± 2.60</td>
<td>6.95 ± 0.28 ( ^{12.20%} )</td>
</tr>
</tbody>
</table>

Values are presented as means ± SEM. N = 5. Significant change from control group at \( ^{*}p < .05 \), \( ^{**}p < .01 \) and \( ^{***}p < .001 \). Significant difference compared with fibrotic model group at \( ^{a}p < .01 \) and \( ^{b}p < .001 \). NS: non-significant. NS: non-significant, a: % of change from control, b: % of change from IRR + CCl₄.

### Figure 4. ALT and AST activities in different groups. Data expressed as Mean ± SD; significant difference vs control group at \( ^{***}p > .001 \), significant difference vs IRR + CCl₄ group at \( ^{*}p < .01 \).

### Figure 5. Levels of total cholesterol and triglycerides in different groups. Data expressed as Mean ± SD; significant difference vs Control group at \( ^{***}p < .001 \), significant difference vs fibrotic group at \( ^{*}p < .01 \).

### Table 4. Total protein, albumin, globulin levels and A/G ratio in different treated groups for two months.

<table>
<thead>
<tr>
<th>Groups</th>
<th>TP (g/dL)</th>
<th>A (g/dL)</th>
<th>G (g/dL)</th>
<th>A/G Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.82 ± 0.34</td>
<td>3.60 ± 0.23</td>
<td>2.60 ± 0.13</td>
<td>1.37 ± 0.06</td>
</tr>
<tr>
<td>AFL extract</td>
<td>5.88 ± 0.41</td>
<td>3.51 ± 0.24</td>
<td>2.42 ± 0.19</td>
<td>1.43 ± 0.07</td>
</tr>
<tr>
<td>CCl₄</td>
<td>4.05 ± 0.15 ( ^{11.0%} )</td>
<td>1.74 ± 0.19 ( ^{25.6%} )</td>
<td>2.30 ± 0.10 ( ^{34.5%} )</td>
<td>0.76 ± 0.09 ( ^{39.5%} )</td>
</tr>
<tr>
<td>IRR + CCl₄</td>
<td>3.32 ± 0.25 ( ^{11.0%} )</td>
<td>1.09 ± 0.09 ( ^{26.5%} )</td>
<td>1.02 ± 0.22 ( ^{30.7%} )</td>
<td>0.5 ± 0.06 ( ^{48.5%} )</td>
</tr>
<tr>
<td>IRR + CCl₄ + AFL</td>
<td>4.70 ± 0.26 ( ^{12.0%} )</td>
<td>2.52 ± 0.16 ( ^{21.0%} )</td>
<td>2.25 ± 0.12 ( ^{27.0%} )</td>
<td>1.4 ± 0.04 ( ^{25.0%} )</td>
</tr>
</tbody>
</table>

Values are presented as means ± SEM. N = 5. Significant change from control group at \( ^{*}p < .05 \), \( ^{**}p < .01 \) and \( ^{***}p < .001 \). Significant difference compared with fibrotic model group at \( ^{a}p < .01 \) and \( ^{b}p < .001 \). NS: non-significant. NS: non-significant, a: % of change from control, b: % of change from IRR + CCl₄.
in IRR + CCl4 + AFL group as compared to those in fibrotic model. Among the evaluation of liver fibrosis markers, the expression levels of TGF-β and collagen-1 were assessed (Figure 6(B)). The CCl4 and IRR + CCl4 groups showed a significant over-expression of TGF-β and collagen-1 (p < .001), compared to that of control. Interestingly, the concurrent oral treatment with AFL extract significantly downregulated TGF-β and collagen-1 expression levels of IRR + CCl4 + AFL group, compared to fibrotic model.

As illustrated in Figure 7(A), the dual toxins caused marked increase in the TIMP-1 protein expression (p > .0001) and decrement of MMPs-9 protein (p > .01), compared to control. Meanwhile, the concurrent administration of AFL extract in IRR + CCl4 + AFL group minimized TIMP-1 protein expression, compared to that of IRR + CCl4 model (p < .001), but still higher than the control (p > .001). Conversely, the expression of MMPs-9 protein was significantly upregulated (p > .01), compared to the fibrotic model and restored near to the normal values of control. Also, western blot analysis confirmed all these changes in Figure 7(B).

Discussion
Liver fibrosis is a pathological reversible condition that involves the fibrogenic response, characterized by scar formation due to increased deposition of ECM leading to fundamental changes in the architecture and functions of liver tissues (Wang et al. 2018). Interestingly, the traditional herbal formulations have elicited a numerous biological activities associated with health effects (Lee et al. 2019). Our previous study evidenced the hepatoprotective effect of AFL extract via antioxidant, anti-inflammatory and anti-apoptotic effects (El-Nashar et al. 2017). In this study, the protective effect of AFL extract was evaluated against c-irradiation and CCl4-induced liver fibrosis of rat model for seven weeks.

The findings of histopathological studies detected an increased number of necrotic hepatocytes, infiltration of inflammatory cells, accompanied with fat steatosis and extensive collagenous formation in IRR + CCl4 model, indicating successful establishment of liver fibrosis model. These results were in accordance with previous reports of El-shawi and her coworkers (El Shawi et al. 2015). Further, the immunohistochemistry results proved over-expression of alpha-smooth muscle actin (α-SMA) in the fibrosis-bearing rats which could be due to the transformation of quiescent HSCs into activated myofibroblasts, leading to upregulation of α-SMA induced by γ-irradiation (Wang et al. 2013) and CCl4 (Hung et al. 2017).
In this study, the dual toxins caused 53% mortality (6/15 rats died) which was decreased to 25% (3/12) in the experimental group. The mortality of rats in the fibrotic model could be attributed to CCl₄ toxicity (Hung et al. 2017). An earlier study reported that the rats exposed to γ-irradiation are more likely at high risk of death due to irradiation-induced immunosuppression (Krishna and Kumar 2005). Of note, all animals treated with the AFL extract alone were not died (0/5).

The abnormal changes of body and liver weights are considered as indicators for liver dysfunction and hepatic toxicity (Mossa et al. 2018). The significant reduction in body weight gain in fibrosis-bearing rats could be due to decrease in food consumption and/or excessive degradation of protein and lipids (Mansour and Mossa 2010). On the other hand, the significant increase of liver weights may be ascribed to the accumulation of collagen and ECM protein deposition in hepatic tissue (Pinzani and Rombouts 2004).

In the present study, the elevated HP and TGF-β levels in fibrotic liver tissues were consistent with upregulation of mRNA expression levels of markers of liver fibrosis; TGF-β and collagen type 1 as well as TIMP-1 protein associated with minimized MMP-9 protein. HP is considered as an index of collagen metabolism and its elevation provides valuable indication of liver fibrosis (Al-Sayed et al. 2014). On the other side, it has been reported that TGF-β expression is overexpressed in all stages of progressive liver fibrosis and attributed to HSCs activation and ECM accumulation, thereby promoting liver fibrosis and cirrhosis (Li et al. 2019). Other researchers reported that the mRNA expression of TGF-β is elevated in patients with liver fibrosis as well as in animals suffered from liver fibrosis (Zhang et al. 2017). The alteration of MMP-9 and TIMP-1 system in fibrosis-bearing rats of our study agreed with a certain study earlier reported that the expression of TGF-β and α-smooth muscle actin were markedly agitated in fibrotic liver model that received myricetin-3-O-α-rhamnoside, indicating its capability to inhibit the pro-fibrotic response. Other investigators as Li et al. (2018) manifested that quercetin significantly inhibited HSC activation, as well as fibrogenic markers such as TGF-β and collagen-1 expression.

The present study clarified that administration of AFL extract significantly attenuated hepatic damage and relieved liver fibrosis in γ-irradiation and CCl₄-induced rat model and considered as a potential herbal drug recommended for countering liver fibrosis.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Notes on contributors

Omana E. El-Shawi, PhD, is a professor of biochemistry at Health Radiation Research Department, National Center for Radiation Research and Technology, Atomic Energy Authority, Cairo, Egypt.

Heba A. S. El-Nashar, PhD, is an assistant professor of pharmacognosy, Faculty of Pharmacy, Ain Shams University, Abbassia, Cairo, Egypt and a researcher at Drug Discovery Research and Development center, Ain Shams University, Egypt.

Sahar S. Abd El-Rahman, PhD, is an associate professor of pathology, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt.

Omayma A. Eldahshan, PhD, is a professor of pharmacognosy, Faculty of Pharmacy, Ain Shams University, Abbassia, Cairo, Egypt and a member of foundation committee of Drug Discovery Research and Development center, Ain Shams University, Egypt.

Abdel Nasser B. Singab, PhD, is a professor of Pharmacognosy, Faculty of Pharmacy, Ain Shams University, Abbassia, Cairo, Egypt and Chief Executive Officer (CEO) of Drug Discovery Research and Development center, Ain Shams University, Egypt.

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