

Antifibrotic effects of *Punica granatum* peels through stimulation of hepatic stellate cell apoptosis in thioacetamide-induced liver fibrosis in rats

Abdel Razik H. Farrag^a, Enayat A. Omara^a, Asmaa F. Galal^b,
Sayed A. El-Toumy^c, Nabila S. Hassan^a, Hafiza A. Sharaf^a, Somaia A. Nada^d

Departments of ^aPathology, ^bToxicology and Narcotics, ^cChemistry of Tannins, ^dPharmacology, National Research Centre, Cairo, Egypt

Correspondence to Abdel Razik H. Farrag, PhD, Pathology Department, Medical Research Division, National Research Centre, 33 El-Buhouth Street, 12622 Dokki, Cairo, Egypt. Tel: +20 233 371 433; fax: +20 233 371 930; e-mail: abdelrazik2000@yahoo.com

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Background/aim

Liver fibrosis is a major global health problem. The present study aimed to evaluate the antioxidant and antifibrogenic potential of *Punica granatum* peels extract against thioacetamide (TAA)-induced hepatic fibrosis.

Materials and methods

Rats were divided into six groups. Group 1 was the control; group 2 was injected with TAA (150 mg/kg, intraperitoneal) (fibrosis group) for 4 weeks; group 3 received *P. granatum* peels extract only (200 mg/kg); group 4 rats were given oral silymarin (50 mg/kg) for 4 weeks after withdrawal of TAA; groups 5 and 6 rats were given oral *P. granatum* peels extract (100 and 200 mg/kg) for 4 weeks after withdrawal of TAA. Fibrosis was assessed histologically and by measuring the hepatic hydroxyproline content. The degree of liver fibrosis was assessed by Masson's trichrome staining and α -smooth muscle actin as the marker of the activated hepatic stellate cells was detected immunohistochemically. Serum markers of liver damage and oxidative stress were also assessed.

Results

The biochemical analyses have shown that *P. granatum* peels extract or silymarin significantly reduced the progression of hepatic fibrosis. The plant extract or silymarin resulted in a significant improvement of liver damage by the reduced levels of serum alanine aminotransferase and alkaline phosphatase. Oral administration of *P. granatum* peels or silymarin has also restored normal levels of malondialdehyde, hydroxyproline content as markers of fibrosis content ($P < 0.05$) in the liver, and retained control activities of endogenous antioxidants such as superoxide dismutase, nitric oxide, and glutathione. The histological evaluation showed that the plant extract or silymarin treatment maintained the architecture of the liver nearly normal and attenuate the accumulation of excessive collagen in the liver fibrosis caused by TAA. We also observed that *P. granatum* peels extract or silymarin-treated rats reduced α -smooth muscle actin.

Conclusion

The obtained results have shown that *P. granatum* peels extract effectively blocked hepatic stellate cell proliferation and they may be beneficial in the treatment of liver fibrosis.

Keywords:

fibrosis, histopathology, immunohistochemistry, *Punica granatum*, thioacetamide

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Introduction

Liver fibrosis is a major global health problem causing ~1.4 million deaths per year [1]. It is the common pathologic result of all chronic liver diseases. Its main causative factors in developing countries are diseases with hepatitis B and C viruses and parasitic infection with *Schistosoma mansoni*, while it can be excessive alcohol consumption in developed countries [2]. In addition, hepatotoxic drugs [antibiotics, carbon tetrachloride, thioacetamide (TAA), and acetaminophen] consequently result in liver fibrosis [3]. The beginning of the hepatotoxic effect of TAA demands metabolic activation and cases of liver fibrosis [4–6]. For the study mechanism of hepatic fibrogenesis and potential antifibrosis we used TAA as the model for liver fibrosis.

The injection TAA in animals gives rise to centrilobular necrosis, apoptosis, and periportal inflammatory cell infiltration and fibrosis in the liver [7]. The development of liver fibrosis is clearly produced with abnormal liver architecture resulting in intense changes of intrahepatic/extrahepatic hemodynamic and finally impairment of the liver function [8]. Liver fibrosis is the result of progressive extracellular matrix (ECM) accumulation, distinguished by scar tissue replacement

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and regenerative nodules occurring in the hepatic perisinusoidal space [9].

Studies over earlier period have focused on the mechanism of fibrosis and fibrogenic cells that create the scarring reaction known as hepatic stellate cells (HSCs). The activation of HSCs and transformation to myofibroblast-like cells then proliferate and produce an ECM with continual chronic inflammation [10]. Oxidative stress, the result of the imbalance between production and clearance of reactive oxidative species (ROS), appears to be a common feature in the different types of liver injuries [11]. In-vitro and in-vivo data have suggested the participation of ROS in the pathogenesis of fibrosis [11,12]. ROS with inflammatory cytokines and growth factors induce HSC activation. Therefore, any interference aimed at reducing the exposure of HSCs to these oxidative and inflammatory stimuli could slow down or inhibit the progression of fibrosis induced by inflammatory cytokines or mediators; activated stellate cells advance hepatic fibrosis and consequently disturb the circulating blood flow in the liver. Physiologically, these pericytes secrete α -smooth muscle actin (α -SMA) that is responsible for connective tissue formation in response to liver injury, and α -SMA is commonly used as a marker of myofibroblast formation [13,14]. Liver lesions from fibrosis are hard to cure, but clinical interventions may block further development or reduce the associated complications [15]. However, current medications for managing hepatofibrosis are limited due to their undesirable side effects [16]. Liver fibrosis could be considered a bidirectional process and could be reversible [17]. The hope is that if antifibrotic therapy can reconstitute the normal balance of the liver, normal function can be restored and clinical appearance may retract. Current and developed approaches primarily target to inhibit the activated HSCs, proliferation, and products as well as to enhance their apoptosis [18].

Medicinal plants have been used from ancient times for the treatment of a large variety of diseases [19] as well as for hepatotoxicity [20]. In fact, this is owing to its lower costs and greater compatibility [21] and being rich in various compounds such as triglycerides, flavonoids, and polyphenols that can protect the liver against damages induced by hepatotoxic drugs [22]. Foods rich in natural antioxidants have been chosen as an agent to prevent and cure liver damage [23]. Pomegranate peel is known for its abundant health-promoting qualities and apparent wound-healing properties [24], anticancer property [25], antiatherosclerotic, antioxidative capacities [26],

antiviral [27], antifungal [28], and antibacterial benefits [29]. This is due to the fact that pomegranate contains large amounts of polyphenols and flavonoids [30]. Furthermore, recent numerous studies have proved the hepatoprotective property of pomegranate that possesses definite hepatoprotective properties, making it a significant therapeutic agent in the treatment of hepatic fibrosis and oxidative damage [31]. The present study was designed to evaluate the antifibrogenic effect of *P. granatum* peels extract on liver fibrosis induced by TAA in rats along with the observation of any potential changes in the biochemical marker, HP as fibrosis markers, histopathological features, and expression of the activated HSCs (SMA).

Materials and methods

Preparation of the *P. granatum* extract

Fresh *P. granatum* L. fruits were collected from Upper Egypt (October 2015). The peels were separated manually from the fruit and then washed with water, cut into small pieces, and sun dried until complete dehydration. Dried peels were ground into a fine powder in a mortar. The dry powder (50 g) was extracted with 300 ml aqueous 70% methanol in a Soxhlet apparatus for 72 h. The extract was filtered and concentrated to dryness under reduced pressure in a rotary evaporator at 40–50°C yielding 14.5% (w/w) plant extract. The obtained *P. granatum* L. peels alcoholic extract was stored at 5°C until usage. The plant extract was suspended in warm, distilled water (100 mg/1 ml) and was given orally through a stomach tube to rats at a dose of 100 and 200 mg/kg [32].

Preparation of drugs

TAA was purchased from Sigma (St Louis, Missouri, USA). Other chemicals and reagents were of high analytical grade and were purchased from standard commercial suppliers. TAA was prepared freshly by dissolving it in sterile distilled water and stirred well until all crystals were dissolved.

Experimental animal procedures

All experimental procedures were performed according to the institutional committee of the animal's care and use guidelines, National Research Centre (Egypt). Male Sprague-Dawley rats (130–150 g body weight) was obtained from the Animal House of the National Research Centre (Egypt). They were maintained under controlled conditions of temperature 37±5°C and kept at 12 h natural day light and dark night cycles. They were provided standard rats feed and water *ad libitum*.

Thirty-six rats were randomly divided into six groups (six rats of each) as follows:

Group 1: served as normal control and received sterile, distilled water only.

Group 2: TAA group in which rats were intraperitoneally injected with TAA (150 mg/kg) for 4 weeks to induce liver fibrosis in rats.

Group 3: rats were orally given *P. granatum* peels (200 mg/kg).

Group 4: silymarin group and given oral dose (50 mg/kg) for 4 weeks after withdrawal of TAA.

Groups 5 and 6: rats were given oral *P. granatum* peels extract (100 and 200 mg/kg, respectively) for 4 weeks after withdrawal of TAA.

After 24 h of the last injection, blood samples were collected from the retro-orbital plexus after light anesthesia with sodium pentobarbital (50 mg/kg, intraperitoneal). Serum was separated by centrifugation at 3000g for 10 min and was used for the assessment of liver functions. Rats were sacrificed by cervical dislocation, and livers were removed. A portion of liver tissue was washed and homogenized to obtain a 20% (w/v) homogenate, which was used for the assessment of oxidative stress and fibrogenic markers. Another portion was placed in formalin for histopathological and immunohistochemical examinations.

Serum biochemistry

Serum concentrations of alanine aminotransferase (ALT) and alkaline phosphatase activity (ALP) were determined according to the methods of Reitman and Frankel [33] and Belfield and Goldberg [34], respectively, using available commercial kits of Biodiagnostics Co. (Cairo, Egypt).

Hepatic oxidative stress markers

The supernatant obtained by centrifugation of the 20% homogenate was used for the assessment of oxidative stress markers. Lipid peroxidation was determined by estimating the level of thiobarbituric acid reactive substances measured as malondialdehyde (MDA), according to the method of Mihara and Uchiyama [35]. Reduced glutathione (GSH) content was determined according to the method of Beutler *et al.* [36] and expressed as mg/g wet tissue. Tissue nitric oxide (NO) metabolites were determined according to the method described by Miranda *et al.* [37] and expressed as $\mu\text{mol/l/g}$ wet tissue. In addition, superoxide dismutase (SOD) activity was determined by a kinetic method using a commercial kit of Ransel, Randox Laboratories Co. (Antrim, UK).

Marker of hepatic fibrosis

Hepatic collagen content was assessed biochemically through the determination of hydroxyproline (HP) concentration, according to the method of Woessner [38]. Results were expressed as $\mu\text{g/g}$ wet tissue.

Histopathological examination

After fixation of liver tissues obtained from rats in the studied groups in 10% formal saline for 24 h, they were washed in tap water. Then, serial dilutions of alcohol were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56° in oven for 24 h. Paraffin wax tissue blocks were prepared for sectioning at 4 μm thickness by rotary microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and were stained by hematoxylin and eosin stains. After that, examination was done using light electron microscope [39].

Masson trichrome stain for collagen fibers

Masson trichrome stain was used for demonstrating the collagen fibers [40].

Immunohistochemical study

Immunostaining for α -SMA was performed on paraffin sections from the livers of all groups. This was done using a primary antiserum to α -SMA (1 : 100) followed by biotinylated horse antimouse antiserum, avidin-biotin complex, and DAB as the chromogen. Smooth muscle was used as positive control specimens. On the other hand, one of the liver specimens was used as negative control by omitting the step of applying the primary antibody. A positive reaction was expressed as a dark brown color in the cytoplasm of hepatocyte stellate cells indicating its activation into myofibroblasts.

Morphometric analysis

The morphometric measurements were done using Leica Quin 500 Image Analyzer (Leica Imaging systems Ltd, Cambridge, UK) in the Pathology Department, National Research Centre, Cairo. The morphometric measurements were carried out with optical magnification of $\times 20$ on hematoxylin and eosin and Masson trichrome stained sections for the measurement of damaged and fibrotic areas. Ten fields were selected randomly for measurements and the results were expressed in μm^2 and SE.

The percent protection with each extract dose was also calculated by the following formula [41]:

$$\% \text{Protection} = \frac{\text{DA}_{\text{TAA}} - \text{DA}_{\text{treated group}}}{\text{DA}_{\text{TAA}}} \times 100,$$

where DA is the damaged area.

Statistical analysis

The results are expressed as mean $\pm$ SE. Multiple comparisons were performed using one-way analysis of variance followed by Tukey–Kramer as a post-hoc test. A *P* value of less than 0.05 was considered statistically significant. All analyses were performed using GraphPad Prism software (version 6) (GraphPad Software, Inc., La Jolla, CA, USA).

Results

P. granatum peels extract treatment attenuated thioacetamide-induced hepatic damage

As compared with the control group, serum ALT levels and ALP activity were significantly elevated in the TAA group. Notably, serum ALT levels and ALP activity were nearly normalized in the *P. granatum* peels extract cotreated group in a dose-dependent manner. Similarly, rats cotreated with silymarin showed nearly normal levels of ALT and ALP activity as compared with the TAA group (Table 1).

P. granatum peels extract treatment attenuated thioacetamide-induced oxidative stress

Hepatic GSH levels and SOD activity were markedly reduced while the liver content of MDA was significantly elevated in the TAA group compared

with the control group. Interestingly, *P. granatum* peels extract or silymarin cotreatment returned both GSH and MDA levels to the nearly normal levels and restored SOD activity, thus protecting against TAA-induced oxidative liver damage. Hepatic levels of NO were markedly increased in rats treated with TAA. Coadministration of *P. granatum* peels extract or silymarin decreased NO levels, dose-dependently, to nearly normal levels as compared with the control group (Table 2).

P. granatum peels extract treatment attenuated thioacetamide-induced hepatic fibrogenesis

Moreover, the antifibrotic effect of *P. granatum* peels extract or silymarin was further confirmed by biochemical determination of HP levels. As seen from Table 2, the increase in HP levels induced by TAA was markedly reduced by *P. granatum* peels extract or silymarin cotreatment in a dose-dependent way. These results confirmed those obtained from histological examination.

Histopathological results

Histology of the liver sections of control rats showed normal hepatic cells with well-preserved cytoplasm, central veins, prominent nucleus, and blood sinusoids (Fig. 1a). Histopathological examination of the livers of TAA group (the fibrosis group) showed disorganization of the normal lobular pattern. Variable degree of degeneration and necrosis were demonstrated starting around the central veins and then progressed to all zones of the hepatic lobules (centrilobular). The affected hepatocytes were vacuolated or fatty degeneration changes, apoptotic having darkly eosinophilic cytoplasm with pyknotic nuclei. Mononuclear cellular infiltration was found between the hepatocytes and marked within the portal areas. Moreover, thick bundles of collagen fibers were bridging the expanded portal areas and central vein and surrounding the lobules (Fig. 1b). *P. granatum* peels extract group (200 mg/kg) revealed

Table 1 Alanine aminotransferase and alkaline phosphatase levels in rats treated with thioacetamide and *P. granatum* extract

Parameters	Groups	
	ALT (U/l)	ALP (U/l)
Control	18 $\pm$ 1.8	96 $\pm$ 4.4
TAA (150 mg/kg)	30 $\pm$ 2.5a	210 $\pm$ 9.2a
Punica (200 mg/kg)	6.5 $\pm$ 0.79b	49 $\pm$ 8.0b
TAA+silymarin	11 $\pm$ 0.89b	50 $\pm$ 7.1b
TAA+punica (100 mg/kg)	16 $\pm$ 1.5b	73 $\pm$ 7.8b
TAA+punica (200 mg/kg)	12 $\pm$ 1.6b	37 $\pm$ 8.9b

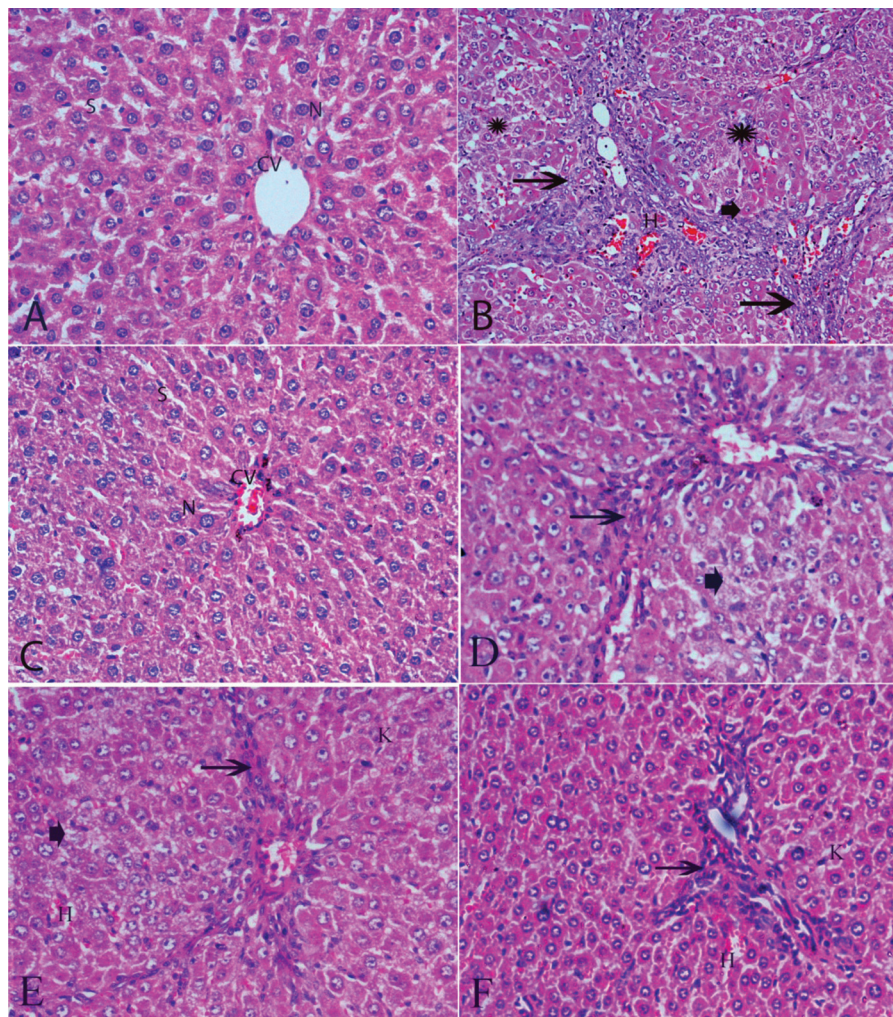
Data presented as mean $\pm$ SE (*n*=6). ANOVA followed by Tukey Honest Significant Differences post-hoc test was used. ALT, alanine aminotransferase; ALP, alkaline phosphatase; ANOVA, analysis of variance; TAA, thioacetamide. ^a*P*<0.05, significantly different from the control group. ^b*P*<0.05, significantly different from the TAA group.

Table 2 Glutathione, superoxide dismutase, malondialdehyde, nitric oxide, and hydroxyproline activities in rats treated with thioacetamide and *P. granatum* extract

Parameters	Groups				
	GSH (μ g/g tissue)	SOD (U/g tissue)	MDA (ng/g tissue)	NO (μ mol/g tissue)	HP (μ g/g tissue)
Control	4.6 $\pm$ 0.29	55.9 $\pm$ 1.52	52 $\pm$ 2.3	1.5 $\pm$ 0.037	126.2 $\pm$ 4.00
TAA (150 mg/kg)	2.8 $\pm$ 0.068a	20.41 $\pm$ 2.84a	114 $\pm$ 5.0a	3.4 $\pm$ 0.32a	243.3 $\pm$ 6.38a
Punica (200 mg/kg)	4.5 $\pm$ 0.046b	76.53 $\pm$ 5.03b	35 $\pm$ 0.9b	0.7 $\pm$ 0.04b	117 $\pm$ 2.3b
TAA+silymarin	4.0 $\pm$ 0.45b	64.38 $\pm$ 5.625b	44 $\pm$ 1.2b	1.4 $\pm$ 0.15b	118.3 $\pm$ 2.404b
TAA+punica (100 mg/kg)	3.4 $\pm$ 0.11b	86.66 $\pm$ 4.6b	63b $\pm$ 5.6b	1.4 $\pm$ 0.087b	121.0 $\pm$ 0.536b
TAA+punica (200 mg/kg)	4.5 $\pm$ 0.35b	58.4 $\pm$ 4.43b	38b $\pm$ 4.4b	0.98 $\pm$ 0.082b	109.7 $\pm$ 3.18b

Data presented as mean $\pm$ SE (*n*=6). ANOVA followed by Tukey-HSD post-hoc test was used. ANOVA, analysis of variance; TAA, thioacetamide. ^a*P*<0.05, significantly different from the control group. ^b*P*<0.05, significantly different from the TAA group.

Figure 1



Micrograph of liver sections stained with hematoxylin and eosin (H&E): (a) the control group showing normal hepatic cells with well-preserved cytoplasm, central veins (CV), prominent nucleus (N) and visible sinusoids (S), (b) thioacetamide (TAA) group showing distortion of the hepatic architecture, extensive intralobular fibrosis of both porto-portal and porto-central bridging fibrosis with collagen septa formation, massive number of inflammatory cells infiltration (arrow), necrotic hepatocytes (arrowhead) with severe ballooning degeneration (star), (c) *P. granatum* peels extract (200 mg/kg) showing normal hepatic architecture and preserved lobular pattern with the CV and rounded nuclei (N), (d) TAA and silymarin showing maintained hepatic architecture with minimal damage, mild necrosis around central vein (arrowhead), mild vacuolar degeneration of hepatocytes (arrowhead), and thin collagenous septa formation with inflammation (arrow), (e) TAA and *P. granatum* peels extract (100 mg/kg) showing partially preserved hepatocytes, moderate necrosis around the central vein (arrowhead), mild vacuolar degeneration of hepatocytes (H), activation of Kupffer cell (K) and the collagen fibers were thinner than those noticed in the TAA group (arrow), and (f) TAA and *P. granatum* peels extract (200 mg/kg) showing minimal damage in the hepatic lobule. The necrotic areas of hepatocytes were replaced by normal cells with thin collagenous septa formation (arrow) (H&E, ×400).

no histopathological changes and the structure was nearly normal (Fig. 1c). In the group treated with TAA and silymarin dose (50 mg/kg), the liver tissue exhibited apparent nearly normal hepatic parenchyma with only a few tiny bundles of collagen fibers and with inflammatory cell infiltration (Fig. 1d). The TAA and *P. granatum* peels extract (100 mg/kg) group showed mild necrosis around the central vein, moderate fatty degeneration of the hepatocytes, and the collagen fibers were thinner than those noticed in the TAA group (Fig. 1e). The *P. granatum* peels extract (200 mg/kg) treated groups showed markedly reduced deposition of fibrous tissue in the liver and tissue morphology nearly similar to the control group (Fig. 1f).

Masson's trichrome staining

Masson trichrome stain is an important marker of liver fibrosis and is used for demonstrating collagen fibers. The connective tissue was demonstrated as a thin layer of collagen fibers in the wall of the central vein and the portal tracts (Fig. 2a). The TAA group displays intense grade of collagen fiber bridge and a pseudolobule structure (Fig. 2b). The *P. granatum* peels extract (200 mg/kg) showed few collagen fibers (Fig. 2c). The TAA group and silymarin (50 mg/kg) showed mild collagen fibers (Fig. 2d). The group that received *P. granatum* peels extract (100 mg/kg) demonstrated less collagen fibers than that observed in the TAA-intoxicated

rat, but was not of normal level (Fig. 2e). By coadministration of *P. granatum* extract (200 mg/kg) the collagen fibers in the treatment groups were markedly reduced in the degree of liver fibrosis (Fig. 2f).

Morphometric analysis

Damaged area

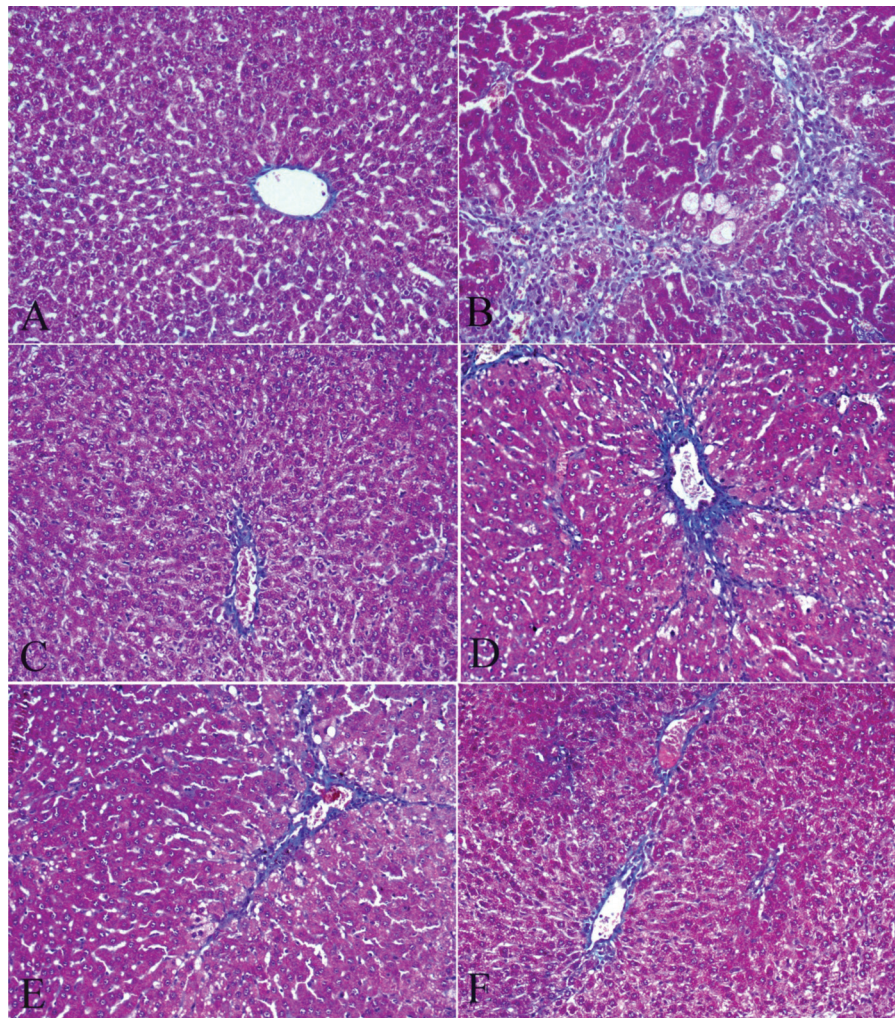
The mean±SE damaged areas of TAA, TAA and silymarine, TAA and *P. granatum* peels extract (100 mg/kg), and TAA and *P. granatum* peels extract (200 mg/kg) are 2561.58±702.44, 779.65±140.17, 667.75±51.98, and 479.72±87.42, respectively (Fig. 3). The data showed significant decrease in the damaged areas of TAA and silymarin, *P. granatum* peels extract (100 mg/kg), and TAA and *P. granatum* peels extract (200 mg/kg) as compared with the TAA group.

The percentages of reduction are 69.56, 73.93 and 81.27%, respectively (Fig. 3).

Fibrotic area

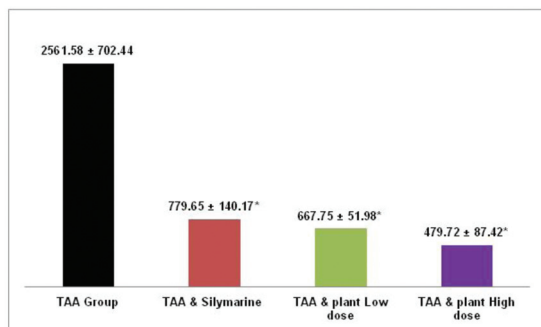
The mean±SE fibrotic areas of control, plant, TAA, TAA and silymarine, TAA and *P. granatum* peels extract (100 mg/kg), and TAA and *P. granatum* peels extract (200 mg/kg) are 71.92±10.53, 81.83±11.35, 2247.22±263.66, 1931.57±193.24, 1052.12±133.52, and 784.38±63.68, respectively (Fig. 4). The data showed significant increase in the fibrotic areas of TAA as compared with the control group. The percentage of elevation is 92.34% and significant decrease in the fibrotic areas of TAA and silymarin, TAA and *P. granatum* peels extract (100 mg/kg), and TAA and *P. granatum* peels extract (200 mg/kg) as compared with the TAA group. The

Figure 2



Micrograph of liver sections stained with Masson's trichrome. Collagen can be visualized by the blue color in stains: (a) the control group showing normal degree of collagen fibers in the central vein and portal tract area; (b) thioacetamide (TAA) group showing extensive intralobular collagen deposition of both porto-portal and porto-central bridging fibrosis apparently as intense blue-stained collagen fibers content in the tissue, (c) *P. granatum* treated group showing nearly normal blue-stained collagen fibers content in the tissue, (d) TAA and silymarin liver shows minimal degree of collagen fibers, (e) TAA and *P. granatum* (100 mg/kg) group showing moderate collagen fibers surrounding the central vein, (f) TAA and *P. granatum* (200 mg/kg) group showing minimal collagen fibers surrounding the central vein (Masson's trichrome, ×400).

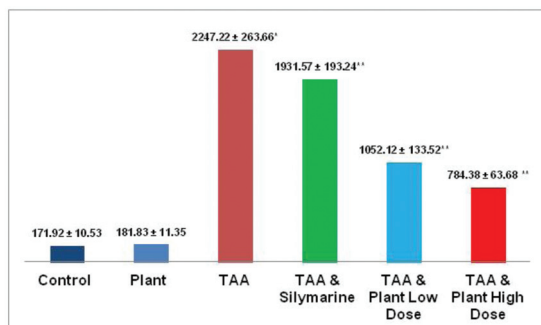
Figure 3



Data presented as mean ± SE
 * Significant decrease as compared with TAA group

Damaged area (µm²) of liver of different groups. Data presented as mean ± SE. *Significant decrease as compared with TAA group.

Figure 4



Data presented as mean ± SE
 * Significant increase as compared with control group
 ** Significant decrease as compared with TAA group

Fibrotic area (µm²) of different groups. Data presented as mean ± SE. *Significant increase as compared with the control group. **Significant decrease as compared with the TAA group.

percentages of reduction are 14.04, 53.18 and 65.50%, respectively.

Immunohistochemical evaluation of α -smooth muscle actin expression

In liver diseases, the major source of matrix proteins produced is to activate HSCs. Therefore, we evaluated immunohistochemical staining of α -SMA as a marker of HSC activation. α -SMA immunoexpression on HSCs were expressed in the form of dark and light stained brown areas in their cytoplasm and was considered a marker of their activation to myofibroblasts. The control group and *P. granatum* extract showed negative expression of α -SMA staining (Fig. 5a and c). The TAA-intoxicated rat demonstrated intense reaction of α -SMA immunostaining in the central and portal tract area. The cytoplasm was stained dark brown color (Fig. 5b). α -SMA expression in the TAA+*P. granatum* peels extract (100 and 200 mg/kg) (Fig. 5e and f) or silymarin-treated groups showed a reduction compared with TAA in a

dose dependent manner indicating inhibition of HSC activation (Fig. 5d).

Discussion

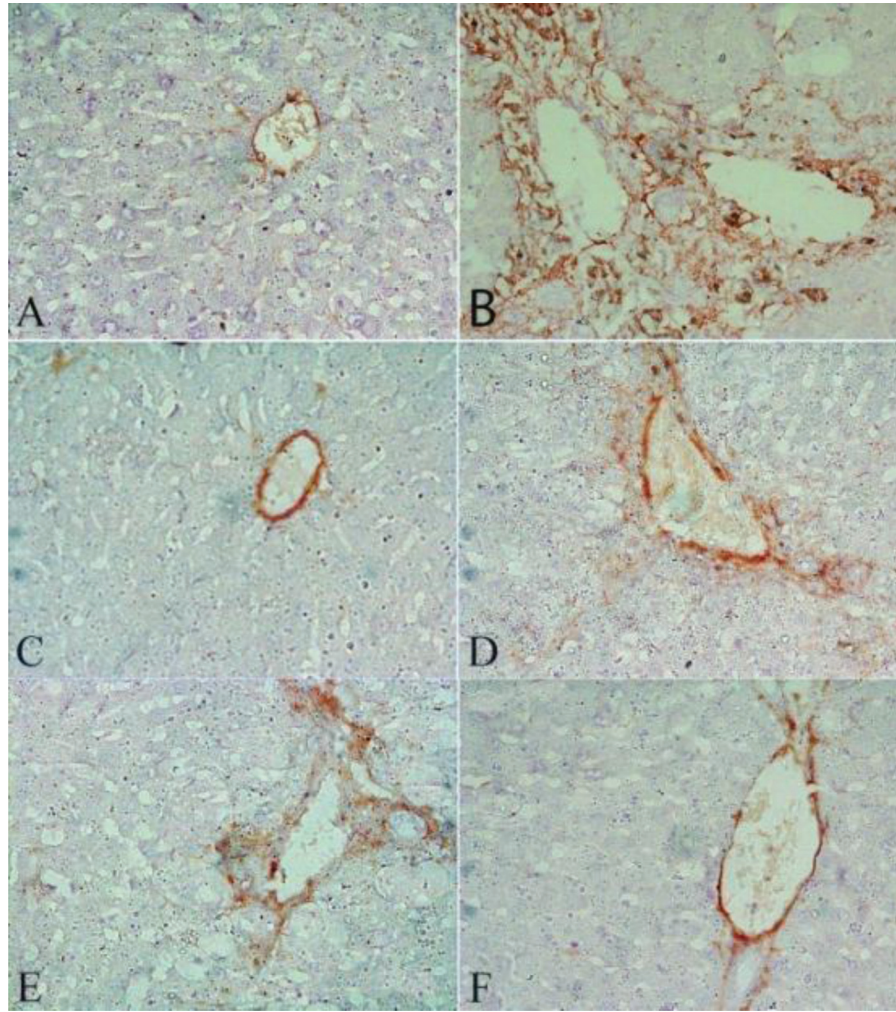
Hepatic fibrosis is known as a massive pathological process that includes various cellular and molecular proceedings that lead to deposition of excess matrix proteins in the extracellular space including collagen [42]. The propose of several antifibrotic treatment effect is particularly studied to downregulate hepatic inflammation, HSC activation, and to increase matrix degradation [43].

The importance of medicinal plants come from a significant achievement therapy of liver fibrosis [44,45]. Traditional plant drugs have been established to be effective in preventing fibrogenesis and other chronic liver injuries which have potential for controlling liver fibrosis, cirrhosis, and hepatocarcinogenesis [46,47]. Flavonoids have beneficial effects as their strong antioxidant characteristic can preserve the body from free radicals and oxidative stress [48]. Besides, many researchers reported using natural antioxidants, such as vitamin E, resveratrol, quercetin and *N*-acetyl cysteine and flavonoids suppression of stellate cell activation [43]. Therefore, flavonoids are convenient to display an essential fundamental biological role, especially due to their capability to scavenge ROS [49].

In the present study, we focused on the antifibrotic effects of *P. granatum* peels by measuring HP; Masson's trichrome staining for collagen content, and immunohistochemical detection of α -SMA as the marker of the activated HSCs. Moreover, the present study was to assess whether dietary *P. granatum* peels extract reduce the progress of liver fibrosis in TAA-intoxicated rats.

The stimulus of fibrosis by TAA occurs *in vivo* and is consider including the producer of oxidative stress [50,51] with the imbalance between fibrosis and antifibrosis signaling pathways. In the current work, the TAA group revealed hepatic damage manifested by significant increase ($P < 0.05$) in serum liver biomarkers (ALT and ALP) when compared with normal control rats. The elevation serum levels of AST and ALT are due to the injury to the impartiality of the liver tissue, since these enzymes normally exist in the cytoplasm and are released to the blood circulation following cellular damage [52]. Hajovsky *et al.* [53] have reported that free radicals produced by TAA affect the cellular permeability of hepatocytes leading to increased levels of serum

Figure 5



Micrograph of liver sections staining with immunohistochemistry for α -smooth muscle actin (α -SMA) can be visualized by the brown color: (a) the control group showing a limited to the wall of the central vein expression of α -SMA; (b) thioacetamide (TAA) group showing intense immunopositive reaction of α -SMA in a fibrous band connecting between portal and central areas as brown color; (c) *P. granatum* group showing normal expression of α -SMA positive staining; (d) TAA and silymarin showing reduction in the areas of α -SMA reaction around the central vein; (e) TAA and *P. granatum* (100 mg/kg) showing moderate α -SMA reaction around the central vein; and (f) TAA and *P. granatum* (200 mg/kg) showing minimal α -SMA reaction around the central vein (α -smooth muscle actin, $\times 400$).

biochemical parameters such as ALT and AST. The treatment with *P. granatum* peel extract or silymarin effectively ameliorated the significant elevation in serum ALT and ALP activities in TAA administered rats. These findings are in agreement with those of Abdel Rahman *et al.* [54] who found that the pomegranate peel extract significantly decreased the damaging influence of CCl_4 on the liver. Silymarin showed a marked protection of serum AST and ALP levels in CCl_4 induced hepatotoxicity due to the high content of flavonoids [55,56]. It was indicated that hepatotoxins with TAA resulted in liver injury by producing free radicals, which then interact with cellular lipids to enhance lipid peroxidation [57]. The elevated MDA level in TAA rats resulted in the current work also agree with this result; however, treatment with *P. granatum* markedly reduced the level of MDA as compared

with the TAA group. These effects were approved by Fadhel and Amran [58] and Ajaikumar *et al.* [59] who show that the tissue lipid peroxidation level was reduced in the *P. granatum* extract treated groups of animals as compared with the TAA group. Besides, silymarin shows noticeable preservation of the liver from raising MDA after being toxicated with CCl_4 [55].

Reduced glutathione is a great endogenous antioxidant system that is located in specially high concentration in the liver, and it is known to have key functions in protective processes. The decreased form of GSH becomes easily oxidized to glutathione disulfide on interaction with free radicals. Progressive induced of free radicals produces the oxidative stress, which leads to damage of macromolecules, for example, lipids and can increase lipid peroxidation *in vivo* [60].

Intoxicated rats with TAA exhibited a significant decrease in the activity of the antioxidant enzymes SOD and GSH concentration as compared with normal control. These are in agreement with Cruz *et al.* [61] who reported that TAA significantly decreased the activity of the antioxidant enzymes (GST, CAT, and SOD) and GSH concentration. Uskokovic-Markovic *et al.* [61] have recorded that TAA resulted in the elevation of oxidative stress, enhancing free radical-mediated damage to proteins, lipids, and DNA. Treatment with *P. granatum* or silymarin exhibited significant increase in the GSH and SOD contents when compared with TAA-induced fibrosis. *P. granatum* and guava leaves have potential antioxidant activity which may be caused due to a lot of phenolic compounds and high antioxidant activity [63,64].

The phytochemical study of pomegranate peel showed the presence of flavonoids, steroids, terpenoids, and tannins [65,66]. The flavonoids have the ability to decrease xenobiotic that produces hepatic damage in animals and oppose the damaging effects of oxidative stress, collaborating with natural systems such as glutathione and other endogenous protective enzymes [67].

In the present study, treatment of TAA group with silymarin showed a significant increase in the activity of the antioxidant enzymes SOD and GSH levels. These results are in agreement with Amin *et al.* [69] and Padhy *et al.* [68] who stated that the treatment of TAA-intoxicated rats with silymarin or/and *Calotropis procera* caused a significant increase in the activity of the antioxidant enzymes. Moreover, silymarin has powerful antioxidant properties because it acts as a scavenger of the free radicals that induce lipid peroxidation, and has great effect on enzyme systems associated with glutathione and superoxide dismutase [70].

The NO radicals play a marked role in producing inflammatory response and their toxicity multiplies only when they interact with $O_2^{\bullet -}$ radicals to form peroxynitrite that causes damages to biomolecules such as proteins, lipids, and nucleic acids [71,72]. In the present results, *P. granatum* extract restores the normal level of NO that increased in the TAA group; however, this result is convenient with the result obtained with the study stated that the pomegranate juice was active and it might have an extremely powerful and novel therapeutic effect for scavenging of NO and may also extend their effects on the regulation of pathological conditions caused by progressive generation of NO and its oxidation product peroxynitrite [73].

Histologically, in our study, TAA injected rats produced severe centrilobular necrosis; hepatocyte vacuolation also scattered inflammatory cell infiltration and fibrosis. These results came in agreement with Ahmed *et al.* [74], who reported that the liver sections of TAA-treated animals exhibit hepatic cells with intense toxicity distinguished by centrilobular necrosis, preportal hepatocyte vacuolation with clearing of cytoplasm, dispersed inflammation, and cell diversion. In addition, these results were nearly similar with numerous past studies, which examine the production of liver fibrosis and cirrhosis by TAA in experimental animals [75,76]. However, in experimental studies, liver fibrosis is commonly induced with TAA, which is readily metabolized to reactive acetamide and TAA-S-oxide. The metabolites formed combine covalently with macromolecules of the hepatic tissue leading to accumulation of fatty acids, damage of proteins and DNA, and formation of ROS. All these compounds impair the endogenous antioxidative system in the liver and are responsible for persistent oxidative stress [75,77].

All these histopathological changes have improvement after treatment with *P. granatum* peel extract or silymarin as compared with the TAA group. The use of *P. granatum* peels extracts reduced the BDL-induced liver fibrosis and improves the liver structure and function [31]. Moreover, these findings are in agreement with Abdel Rahman *et al.* [54], Sadia *et al.* [78], and Ibrahim [79] who found that administration of pomegranate peel extract significantly reduced the liver damage and able to improve hepatic steatosis induced by CCl_4 . The pomegranate peels extract significantly suppress ferric nitrilotriacetate produced oxidative stress and also prevent necrosis and other pathological changes and preserve the hepatic architecture [80]. Similar hepatoprotective effects have been reported with pomegranate peels extract, which inhibited CCl_4 , or pentachlorophenol-induced oxidative stress and hepatic injury [58,81].

Pomegranate juice and peel, seeds extracts have antioxidant potential due to high the content of polyphenolic compounds [82] and possess a potent antioxidant activity [83], and due to their active compounds that have certain electron donors, which can react with the free radicals to change them to more stable products and terminate the radical chain reaction [54,78,79]. In addition, pomegranate peels fundamentally have phenolics, inclusive mainly of hydrolysable tannins (ellagitannins), such as oligomers and punicalagin/punicalin [84]. The treatment with silymarin exhibited improvement of the liver tissue and absence of centrilobular or bridging necrosis [70].

In the present study, increases of collagen content in TAA-treated animals were observed in all liver tissues. HSC, which is the central mediator in the pathogenesis of fibrosis, are known to be activated by free radicals and increase the ECM with collagen [85]. Mean area percent of collagen fibers was used as an index for assessing the extent of liver fibrosis [86]. However, *P. granatum* peel extracts markedly decreased the collagen fiber content as compared with the fibrosis group induced by TAA. Moreover, there was statistically significant difference between the TAA group (fibrosis group) and the treatment group (*P. granatum* peels 100 and 200 mg/kg) based on quantitative morphometric analysis results.

The treatment of mice with CCl₄ and pomegranate peel caused a detectable decrease of the collagen fibers; these effects could be related to its antioxidant, antifibrotic, and antiapoptotic properties [85]. Various studies proved that oxidative stress plays a vital role in liver fibrosis [86]. Antioxidants are effective for preventing liver fibrogenesis [87]. Moreover, pomegranate peel antioxidant and antifibrotic properties may be having powerful therapeutic value in preserving liver tissues from fibrosis and oxidative injury [85]. Therefore, *P. granatum* peels treatment may be helpful as therapy with consideration to antihepatofibrotic properties.

It is commonly known that HSC activation plays a pivotal role in the process of hepatic fibrogenesis, and α -SMA is a marker of activated HSCs [88]. In the present study, immunohistochemical observations of α -SMA was increased in rats injected with TAA and after treated with *P. granatum* peel extract or silymarin noticeably suppressed α -SMA; however, this indicated suppression of the activation of HSCs.

The antioxidant influence of flavonoids in pomegranate peels extract increases the process of regeneration. This might be due to destruction of free radicals, supplying a competitive substrate for unsaturated lipids in the membrane and/or progress the restoration mechanism of damaged cell membrane [89]. Additionally, flavonoid compounds have a stronger antioxidative activity and can exert their protective role by regulating cell apoptosis; these compounds can inhibit normal hepatic cell apoptosis while accelerating apoptosis of tumor cells and necrotic hepatic cells. Flavonoid compounds also protect liver against damage via modulation of cell mitosis and proliferation as well as the secretion of enzymes against platelets during coagulation and inflammation [90–92].

Conclusion

We have confirmed that *P. granatum* peels extract has obvious hepatoprotective and antifibrotic powerful effects against TAA-induced fibrosis using an in-vivo model. This helped to clarify the potential antioxidant efficacy of *P. granatum* peels extract and its ability to suppress HP, collagen content as well as α -SMA. Furthermore, liver function including that of ALT, ALP, and antioxidant enzymes (GSH, SOD, and NO) were improved after *P. granatum* peels extract treatment. Besides, the obtained results showed that *P. granatum* peels extract effectively blocked HSC proliferation and they may be beneficial in therapeutic liver fibrosis. In addition, we demonstrated the potent antifibrotic role of flavonoid compounds existent in *P. granatum* peels extract providing a theoretical basis for its clinical application and indicating an alternative method for the clinical treatment of liver fibrosis.

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Conflicts of interest

There are no conflicts of interest.

References

- 1 Liu T, Wang X, Karsdal MA, Leeming DJ, Genovese F. Molecular serum markers of liver fibrosis. *Biomark Insights* 2012; 7:105–117.
- 2 Alshawsh MA, Abdulla MA, Ismail S, Amin ZA. Hepatoprotective effects of *Orthosiphon stamineus* extract on thioacetamide-induced liver cirrhosis in rats. *Evid Based Complement Altern Med* 2011; 2011:103039.
- 3 Saleem TS, Chetty SM, Ramkanth S, Rajan VS, Kumar KM, Gauthaman K. Hepatoprotective herbs-a review. *Int J Res Pharmaceut Sci* 2010; 1:1–5.
- 4 Wang T, Fontenot RD, Soni MG, Bucci TJ, Mehendale HM. Enhanced hepatotoxicity and toxic outcome of thioacetamide in streptozotocin-induced diabetic rats. *Toxicol Appl Pharmacol* 2000; 166:92–100.
- 5 Ramaiah SK, Apte U, Mehendale HM. Cytochrome P4502E1 induction increases thioacetamide liver injury in diet-restricted rats. *Drug Metab Dispos* 2001; 29:1088–1095.
- 6 Chilakapati J, Korrapati MC, Hill RA, Warbritton A, Latendresse JR, Mehendale HM. Toxicokinetics and toxicity of thioacetamide sulfoxide: a metabolite of thioacetamide. *Toxicology* 2007; 230:105–116.
- 7 Ledda-Columbano GM, Coni P, Curto M, Giacomini L, Faa G. Induction of two different modes of cell death, apoptosis and necrosis, in rat liver after a single dose of thioacetamide. *Am J Pathol* 1991; 139:1099–1109.
- 8 Kumar V, Abbas AK, Fausto N. Robbins and Cotran pathologic basis of disease. Philadelphia, PA: Elsevier Saunders; 2004.
- 9 Kisseleva T, Brenner DA. Anti-fibrogenic strategies and the regression of fibrosis. *Best Pract Res Clin Gastroenterol* 2011; 25:305–317.
- 10 Tacke F, Weiskirchen R. Update on hepatic stellate cells: pathogenic role in liver fibrosis and novel isolation techniques. *Expert Rev Gastroenterol Hepatol* 2012; 6:67–80.
- 11 Sánchez-Valle V, Chávez-Tapia NC, Uribe M, Méndez-Sánchez N. Role of oxidative stress and molecular changes in liver fibrosis: a review. *Curr Med Chem* 2012; 19:4850–4860.
- 12 Liedtke C, Luedde T, Sauerbruch T, Scholten D, Streetz K, Tacke F, et al. Experimental liver fibrosis research: update on animal models, legal issues and translational aspects. *Fibrogenesis Tissue Repair* 2013; 6:19–42.
- 13 Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest* 2005; 115:209–218.

- 14 Juakiem W, Torres DM, Harrison SA. Nutrition in cirrhosis and chronic liver disease. *Clin Liver Dis* 2014; 18:179–190.
- 15 Collins P, Ayres L, Valliani T. Drug therapies in liver disease. *Clin Med* 2013; 13:585–591.
- 16 Van der Meer AJ, Veldt BJ, Feld JJ, Wedemeyer H, Dufour JF, Lammert F, *et al.* Association between sustained virological response and all-cause mortality among patients with chronic hepatitis C and advanced hepatic fibrosis. *JAMA* 2012; 308:2584–2593.
- 17 Friedman SL. Evolving challenges in hepatic fibrosis. *Nat Rev Gastroenterol Hepatol* 2010; 7:425–436.
- 18 Zhao W, Li JJ, Cao DY, Li X, Zhang LY, He Y. Intravenous injection of mesenchymal stem cells is effective in treating liver fibrosis. *World J Gastroenterol* 2012; 18:1048–1058.
- 19 Khosravi-Boroujeni H, Mohammadifard N, Sarrafzadegan N, Sajjadi F, Maghroun M, Khosravi A, *et al.* Potato consumption and cardiovascular disease risk factors among Iranian population. *Int J Food Sci Nutr* 2012; 63:913–920.
- 20 Heidarian E, Rafieian-Kopaei M. Protective effect of artichoke (*Cynara scolymus*) leaf extract against lead toxicity in rat. *Pharm Biol* 2013; 51:1104–1109.
- 21 Abdollahy F, Ziaei H, Shabankhani B, Azadbakht M. Effect of essential oils of *Artemisia aucheri* Boiss. *Zataria multiflora* Boiss and *Myrtus communis* L. on *Trichomonas vaginalis*. *Iran J Pharmaceut Res* 2004; 3(Supp 2):35–35.
- 22 Galisteo M, Suarez A, Montilla MP, Utrilla MD, Jimenez J, Gil A, *et al.* Antihepatotoxic activity of *Rosmarinus tomentosus* in a model of acute hepatic damage induced by thioacetamid. *Phytother Res* 2000;14: 522–526.
- 23 Morisco F, Vitaglione P, Amoruso D, Russo B, Fogliano V, Caporaso N. Foods and liver health. *Mol Aspects Med* 2008; 29:144–150.
- 24 Chidambara MK, Reddy VK, Veigas JM, Murthy UD. Study on wound healing activity of *Punica granatum* peel. *J Med Food* 2004; 7:256–259.
- 25 Jeune MA, Kumi-Diaka J, Brown J. Anticancer activities of pomegranate extracts and genistein in human breast cancer cells. *J Med Food* 2005; 8:469–475.
- 26 Tzulkar R, Glazer I, Bar-Ilan I, Holland D, Aviram M, Amir R. Antioxidant activity, polyphenol content and related compounds in different fruit juices and homogenates prepared from 29 different pomegranate accessions. *J Agric Food Chem* 2007; 55:9559–9570.
- 27 Zhang J, Zhan B, Yao X, Gao Y, Shong J. Antiviral activity of tannin from the pericarp of *Punica granatum* L. against genital Herpes virus *in vitro*. *Zhongguo Zhong Yao Za Zhi* 1995; 20:556–558.
- 28 Dutta BK, Rahman I, Das TK. Antifungal activity of Indian plant extracts. *Mycoses* 1998; 41:535–536.
- 29 Prashanth D, Asha M, Amit A. Antibacterial activity of *Punica granatum*. *Fitoterapia* 2001; 72:171–173.
- 30 Go? mez-Caravaca AM, Verardo V, Toselli M, Segura-Carretero A, Ferna? ndez-Gutierrez A, Caboni MF. Determination of the major phenolic compounds in pomegranate juices by HPLC–DAD–ESI–MS. *J Agric Food Chem* 2013; 61:5328–5337.
- 31 Toklu HZ, Dumlu MU, Sehirli O, Ercan F, Gedik N, Gökmen V, *et al.* Pomegranate peel extract prevents liver fibrosis in biliary-obstructed rats. *J Pharm Pharmacol* 2007; 59:1287–1295.
- 32 Gozlekci S, Saracoglu O, Onursal E, Ozgen M. Total phenolic distribution of juice, peel, and seed extracts of four pomegranate cultivars. *Pharmacogn Mag* 2011; 7:161–164.
- 33 Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. *Am J Clin Path* 1957; 58:56–61.
- 34 Belfield A, Goldberg DM. Colorimetric method for determination of alkaline phosphatase. *Enzyme* 1971; 12:561.
- 35 Mihara M, Uchiyama M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal Biochem* 1978; 86:271–278.
- 36 Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. *J Lab Clin Med* 1963; 61:882–890.
- 37 Miranda KM, Espey MG, Wink DA. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide* 2001; 5:62–71.
- 38 Bancroft J, Gamble M. Theory and practice of histological techniques. 6th ed. London, UK: Churkhil Livinstone; 2008. pp. 433–P469.
- 39 Avwioro OG. Staining reactions of microwave processed tissues compared with conventional paraffin wax processed tissues. *Eur J Exp Bio* 2011; 1:57–62.
- 40 Suzuki Y, Hayashi M, Ito M, Yamagami I. Antiulcer effects of cetraxate on various experimental gastric ulcers in rats. *Jpn J Pharmacol* 1976; 26: 471–480.
- 41 Iredale J. Defining therapeutic targets for liver fibrosis: exploiting the biology of inflammation and repair. *J Pharma Res* 2008; 58: 129–136.
- 42 Li D, Friedman SL. Liver fibrogenesis and the role of hepatic stellate cells: new insights and prospects for therapy. *J Gastroenterol Hepatol* 1999; 14:618–633.
- 43 Shih CC, Wu YM, Lin WC. Aqueous extract of *Aroectochilus formosanus* attenuate hepatic fibrosis induced by CCl₄ in rats. *Phytomedicine* 2005; 12:453–460.
- 44 Gui SY, Wei W, Wang H, Wu WYL, Sun WB, Chen CY, *et al.* Effects and mechanism of crude astragalosides fraction on liver fibrosis in rats. *J Ethnopharmacol* 2006; 103:154–159.
- 45 Yao XX, Jiang SL, Tang YN, Yao DM, Yao X. Efficacy of Chinese medicine Yi-gan-kang granule in prophylaxis and treatment of liver fibrosis in rats. *World J Gastroenterol* 2005; 11:2583–2590.
- 46 Inao M, Mochida S, Matsui A, Eguchi Y, Yulutuz Y, Wang Y, *et al.* Japanese herbal medicine Inchin-ko-to as a therapeutic drug for liver fibrosis. *J Hepatol* 2004; 41: 584–591.
- 47 Bors W, Heller W, Michel C, Stettmaier K. Handbook of antioxidants. In: Cadenas E, Packer L, editor. New York, NY: Marcel Dekker; 1996. pp. 409–466.
- 48 Pietta PG, Simonetti P, Roggi C, Brusamolino A, Pellegrini N, Maccarini L, *et al.* Natural antioxidants and food quality in atherosclerosis and cancer prevention. In: Kumpulainen JT, Salonen JT, editors. Cambridge, UK: Royal Society of Chemistry; 1996. pp. 249–255.
- 49 Abdel Salam OM, Mohammed NA, Sleem AA, Farrag AR. The effect of antidepressant drugs on thioacetamide-induced oxidative stress. *Eur Rev Med Pharmacol Sci* 2013; 17:735–744.
- 50 Al-Attar AM. Attenuating effect of *Ginkgo biloba* leaves extract on liver fibrosis induced by thioacetamide in mice. *J Biomed Biotechnol* 2012; 2012:761450.
- 51 Shim JY, Kim MH, Kim HD, Ahn JY, Yun YS, Song JY. Protective action of the immunomodulator ginsan against carbon tetrachloride-induced liver injury via control of oxidative stress and the inflammatory response. *Toxicol Appl Pharmacol* 2010; 242:318–325.
- 52 Hajovsky H, Hu G, Koen Y, Sarma D, Cui W, Moore DS, *et al.* Metabolism and toxicity of thioacetamide and thioacetamide S-oxide in rat hepatocytes. *Chem Res Toxicol* 2012; 25:1955–1963.
- 53 Abdel Rahman MK, Ashraf A, El-Megeid A. Hepatoprotective effect of soapworts (*Saponaria officinalis*), pomegranate peel (*Punica granatum* L) and cloves (*Syzygium aromaticum* linn) on mice with CCl₄ hepatic intoxication. *WJC* 2006; 1:41–46.
- 54 Wills PJ, Asha VV. Preventive and curative effect of *Lygodium flexuosum* (L.) Sw. on carbon tetrachloride induced hepatic fibrosis in rats. *J Ethnopharmacol* 2006; 107:7–11.
- 55 Wagner H. Antihepatotoxic flavonoids. In: Cody V, Middleton E Jr, Hardborne JB, editors. Plant flavonoids in biology and medicine; biochemical, pharmacological and structure-activity relationships. New York, NY: Alan R. Ed. Liss Inc; 1986. pp. 545–558.
- 56 Fadhel ZA, Amran S. Effects of black tea extract on carbon tetrachloride-induced lipid peroxidation in liver, kidneys, and testes of rats. *Phytother Res* 2002; 16:S28–S32.
- 57 Chidambara KN, Murthy KN, Jayaprakash GK, Singh RP. Studies on antioxidant activity of pomegranate (*Punica granatum*) peel extract using in vivo models. *J Agric Food Chem* 2002; 50: 4791–4795.
- 58 Ajai Kumar KB, Asheef M, Babu BH, Padikkala J. The inhibition of gastric mucosal injury by *Punica granatum* L. (pomegranate) methanolic extract. *J Ethnopharmacol* 2005; 96: 171–176.
- 59 Sinclair AJ, Barnett AH, Lunie J. Free radical and auto-oxidant systems in health and disease. *J Appl Med* 1991; 17:409–412.
- 60 Cruz A, Padillo FJ, Torres E, Navarrete CM, Muñoz-Castañeda JR, Caballero FJ, *et al.* Melatonin prevents experimental liver cirrhosis induced by thioacetamide in rats. *J Pineal Res* 2005; 39: 143–150.
- 61 Uskokovic-Markovic S, Milenkovic M, Topic A, Kotur-Stevuljevic J, Stefanovic A, Antic-Stankovic J. Protective effects of tungstophosphoric acid and sodium tungstate on chemically induced liver necrosis in wistar rats. *J Pharm Sci* 2007; 10:340–349.
- 62 Tachakittirungrod S, Okonogi S, Chowwanapoonpohn S. Study on antioxidant activity of certain plants in Thailand: Mechanism of antioxidant action of guava leaf extract. *Food Chem* 2007; 103:381–388.

- 63 Osman M, Ahmed M, Mahfouz S, Elaby S. Biochemical studies on the hepatoprotective effects of pomegranate and guava ethanol extracts. *N Y Sci J* 2011; 4:27–41.
- 64 Carini R, Comoglio A, Albano E, Poli G. Lipid peroxidation and irreversible damage in the rat hepatocyte model: protection by the silybin-phospholipid complex IdB 1016. *Biochem Pharmacol* 1992; 43:2111–2115.
- 65 Paya M, Ferrandiz ML, Sanz MJ, Alcaraz MJ. Effects of phenolic compounds on bromobenzene-mediated hepatotoxicity in mice. *Xenobiotica* 1993; 23:327–333.
- 66 Kadarian C, Broussalis AM, MinoLopez J, Gor-zalczy PS, Ferraro P, Acevedo G. Hepatoprotective activity of *Achyrocline satureioides* (Lam) D. C. *Pharm Res* 2002; 45:57–61.
- 67 Padhy B, Srivastava A, Kumar V. Calotropis procera latex affords protection against carbon tetrachloride induced hepatotoxicity in rats. *J Ethnopharmacol* 2007; 113:498–502.
- 68 Ghosh A, Ghosh T, Jain S. Silymarin: a review on the pharmacodynamics and bioavailability enhancement approaches. *J Pharm Sci Technol* 2010; 2:348–355.
- 69 Gouthamchandra K, Mahmood R, Manjunatha H. Free radical scavenging, antioxidant enzymes and wound healing activities of leaves extracts from *Clerodendrum infortunatum* L. *Environ Toxicol Pharmacol* 2010; 30:11–18.
- 70 Gulcin I, Oktay M, Kufrevioglu OI, Aslan A. Determination of antioxidant activity of lichen *Cetraria islandica* (L) Ach. *J Ethnopharmacol* 2002; 79:325–329.
- 71 Al-Olayan E, Manal M, El-Khadragy F, Metwally DM, Abdel Moneim AE. Protective effects of pomegranate (*Punica granatum*) juice on testes against carbon tetrachloride intoxication in rats. *BMC Complement Altern Med* 2014; 14:164–172.
- 72 Ahmed KM, Saleh EM, Sayed EM, Shalaby KA. Anti-inflammatory effect of different propolis extracts in thioacetamide-induced hepatotoxicity in male rat. *Aust J Basic Appl Sci* 2012; 6:29–40.
- 73 Mir Al, Kumar B, Tasduq SA, Gupta DK, Bhardwaj S, Johri RK. Reversal of hepatotoxin-induced pre-fibrogenic events by *Emblia officinalis* – a histological study. *Indian J Exp Biol* 2007; 45:626–629.
- 74 Li JF, Chen BC, Lai DD, Jia ZR, Andersson R, Zhang B, et al. Soy isoflavone delays the progression of thioacetamide-induced liver fibrosis in rats. *Scand J Gastroenterol* 2011; 46:341–349.
- 75 Baskaran N, Periyasam V, Venkatraman AC. Investigation of antioxidant, anti-inflammatory and DNA-protective properties of eugenol in thioacetamide-induced liver injury in rats. *Toxicology* 2010; 268:204–212.
- 76 Sadia H, Akter QS, Afroz R, Siddika T. Effect of *Punica granatum* (pomegranate) on serum ALT and AST in carbon tetrachloride induced liver damage in Wistar albino rats. *JBSP* 2016; 11:23–28.
- 77 Ibrahim I. Efficiency of pomegranate peel extract as antimicrobial, antioxidant and protective agents. *World J Agri Sci* 2010; 6:338–344.
- 78 Kaur G, Jabbar Z, Athar M, Alam S. Punica granatum (pomegranate) flower extract possesses potent antioxidant activity and abrogates Fe-NTA induced hepatotoxicity in mice. *Food Chem Toxicol* 2006; 44:984–993.
- 79 Agha FE, Hassannane MM, Omara EA, Hasan AM, El-Toumy SA. Protective effect of *P. granatum* peel extract against pentachlorophenol-induced oxidative stress, cytogenetic toxicity and hepatic damage in rats. *Australian J Basic Appl Sci* 2013; 7:853–864.
- 80 Seeram NP, Adams LS, Henning SM, Niu Y, Zhang Y, Nair MG, et al. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. *J Nutr Biochem* 2005; 16:360–367.
- 81 Gil MI, Tomás-Barberán FA, Hess-Pierce B, Holcroft DM, Kader AA. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *J Agric Food Chem* 2000; 48:4581–4589.
- 82 Sadeghipour A, Eidi M, Ilchizadeh Kavani A, Ghahramani R, Shahabzadeh S, Anissian A. Lipid lowering effect of *Punica granatum* L. peel in high lipid diet fed male rats. *Evid Based Complement Alternat Med* 2014; 2014:5–11.
- 83 Tahan V, Ozaras R, Canbakan B, Uzun H, Aydin S, Yildirim B, et al. Melatonin reduces dimethylnitrosamine-induced liver fibrosis in rats. *J Pineal Res* 2004; 37:78–84.
- 84 Toyoki Y, Sasaki M, Narumi S, Yoshihara S, Morita T, Konn M. Semiquantitative evaluation of hepatic fibrosis by measuring tissue hydroxyproline. *Hepatogastroenterology* 1998; 45:2261–2264.
- 85 Wasser S, Ho JM, Ang HK, Tan CE. Salvia miltiorrhiza reduces experimentally-induced hepatic fibrosis in rats. *J Hepatol* 1998; 29: 760–771.
- 86 Kisseleva T, Brenner DA. Mechanisms of fibrogenesis. *Exp Biol Med* (Maywood) 2008; 233:109–122.
- 87 Yuan LP, Chen FH, Ling L, Bo H, Chen ZW, Li F, et al. Protective effects of total flavonoids of *Bidens bipinnata* L. against carbon tetrachloride-induced liver fibrosis in rats. *J Pharm Pharmacol* 2008; 60:1393–1402.
- 88 Dong M, Hong T, Liu S, Zhao J, Meng Y, Mu J. Hepatoprotective effect of the flavonoid fraction isolated from the flower of *Inula britannica* against d-galactosamine-induced hepatic injury. *Mol Med Rep* 2013; 7:1919–1923.
- 89 Woessner JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys* 1961; 93:440–447.
- 90 Amin ZA, Bilgen M, Alshawsh MA, Ali HM, Hadi AH, Abdulla MA. Protective role of *phyllanthus niruri* extract against thioacetamide induced liver cirrhosis in rat model. *Evid Based Complement Alternat Med* 2012; 2012:9.
- 91 Salwe KJ, Sachdev DO, Bahurupi Y, Kumarappan M. Evaluation of antidiabetic, hypolipidemic and antioxidant activity of hydroalcoholic extract of leaves and fruit peel of *Punica granatum* in male Wistar albino rats. *J Nat Sci Biol Med* 2015; 6:56–62.
- 92 Poli G. Pathogenesis of liver fibrosis: role of oxidative stress. *Mol Aspects Med* 2000; 21:49–98.