REGRESSION OF LIVER FIBROSIS BY *PUNICA GRANATUM* PEEL EXTRACT IN THE EXPERIMENTAL MODEL

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ABSTRACT

This study was designed to investigate the efficacy of *Punica granatum* peel methanolic extract against TAA-induced liver fibrosis in rats. Liver enzymes, plasma fibrinogen, serum hepatocyte growth factor levels, reduced glutathione content were estimated. Hepatic NQO1 and BCL-2 gene expression levels were detected by semiquantitative RT-PCR. In addition, histopathological investigation of liver tissue sections was carried out. The positive control group (TAA-treated) showed significant elevation in the activity of liver enzymes, bilirubin and serum hepatocyte growth factor levels. On the other hand, this group revealed significant reduction in plasma fibrinogen level and hepatic reduced glutathione content. Additionally, significant downregulation in hepatic NQO1 and BCL-2 gene expression levels were detected in the positive control group as compared to the negative control group. Histopathological investigation of liver tissue sections of rats in the positive control group revealed many fibrotic features. In contrast, *Punica granatum* -treated group showed significant depletion in serum liver enzymes activity, bilirubin and hepatocyte growth factor levels accompanied with significant elevation in plasma fibrinogen level and hepatic reduced glutathione content to near normal values. Moreover, this group showed dramatic upregulation in hepatic NQO1 and BCL-2 gene expression levels as compared to the positive control group interestingly, treatment with *Punica granatum* caused marked improvement in the histological feature of liver tissue. This study indicated the promising therapeutic potential of *Punica granatum* peel extract in the regression of liver fibrosis. This effect could be attributed to its antioxidant properties, antifibrotic, and antiapoptotic activity.
Keywords: liver fibrosis, Punica granatum, antioxidant, apoptosis, Rats.

INTRODUCTION
Liver is the main organ responsible for multitude of essential functions and plays an important role in the metabolism of foreign compounds entering the body. Human beings are exposed to these compounds through consumption of contaminated food or during exposure to chemical substances in the occupational environment. These foreign compounds produce variety of toxic manifestations particularly in the liver\(^1\). In Egypt, liver diseases are one of the most prominent killers specifically fibrosis, hepatitis C virus (HCV) and cirrhosis that alter the functions of the liver\(^2\).

Liver fibrosis is a wound healing process that results from chronic liver damage, such as alcohol abuse, chronic hepatitis, nonalcoholic steatohepatitis and overload of metal ions\(^3,4\). Liver fibrosis is the final pathway for most chronic liver diseases and is the main reason for increased mortality in affected patients. Fibrotic tissue is characterized by loss of normal structure, replacement of blood vessels and other parenchymal structures by increasingly stable extracellular matrix (ECM), in which type I fibrillar collagen is the major component\(^5\).

In the presence of hepatic injury, hepatic stellate cells (HSCs) become activated and transform into proliferative myofibroblast-like cells, which are the major source of ECM. In general, liver fibrosis is an imbalance between the synthesis and degradation of ECM and it is reversible before turning into cirrhosis, which is the irreversible end stage leading to liver failure\(^6\). Despite significant progress in our understanding of fibrogenesis, injury stimuli process, inflammation, HSC activation and ECM expression, there is still no standard treatment for liver fibrosis.

Administration of thioacetamide (TAA) in rodents is a widely used model to study mechanisms of hepatic injury. TAA causes hepatocyte injury via biotransformation of TAA by cytochrome P450 2E1 (CYP2E1) enzymes located in the microsomes of liver cells into a highly reactive toxic intermediate known as thioacetamide sulphur dioxide\(^7\). This toxic metabolite induces hepatotoxicity in different grades of liver damage including fibrosis nodular cirrhosis, production of pseudolobules, proliferation of hepatic cells, and necrosis of parenchyma cells in the experimental animals\(^8\).

Punica granatum (Pomegranate) is one of the oldest edible fruit belongs to the Punicaceae family\(^9\). It is extensively cultivated in the Mediterranean area and mostly in Near and Far
East countries. Pomegranate has been shown to reduce oxidative stress mediators clearly indicating its antioxidant ability which is attributed to its diverse phenolic compounds\textsuperscript{[10]} These include punicalagin isomers, ellagic acid derivatives and anthocyanins\textsuperscript{[11]} which are known for their properties in scavenging free radicals and inhibiting lipid oxidation\textsuperscript{[12]}.

The present study aimed to investigate the efficacy of \textit{Punica granatum} peel methanolic extract against liver fibrosis induced in rats by thioacetamide. The influence of \textit{Punica granatum} on liver functions and hepatocyte growth factor was determined. Besides, the possible effect of \textit{Punica granatum} on the anti-apoptotic gene (BCL-2) and glutathione as well as NQO1 gene, as key players in oxidative defense, was investigated. In addition, the effect of \textit{Punica granatum} extract on the histopathological feature of the liver was examined.

**MATERIALS AND METHODS**

**MATERIALS**

**Chemicals**

Thioacetamide (TAA) was purchased from Sigma Aldrich.Chemical.Co., (St.Louis,MO, USA) as pure crystals. It was dissolved in saline and freshly prepared prior to each injection. All other chemicals and solvents were of analytical grade and were obtained from commercial sources.

**Preparation of \textit{Punica granatum} peel methanolic extract:**

One kilogram of \textit{Punica granatum} peel was separated from seeds of \textit{Punica granatum} fruits. The peel was cut into small pieces and blended with 2000 ml of methanol 70\% using electric blender. Then, the mixture was left for 72 hrs and filtered using filter paper. The total filtrate was then concentrated to dryness in \textit{Vacuo} at 40\°C in order to render the MeOH extract (130.35g)\textsuperscript{[13]}.

**Experimental Design**

Forty adult male Wistar rats weighing 150-200 g were obtained from the Animal House Colony of the National Research Centre, Cairo, Egypt. The animals were housed in polypropylene cages in an environmentally controlled clean air room with a temperature of 25±1\°C, an alternating 12h light/12h dark cycle, a relative humidity of 60 \± 5\% and free access to tap water and a standard rodent chow. Rats were allowed to adapt to these conditions for 2 weeks before beginning the experimental protocol. The experimental protocol was approved by the Ethical Committee for Medical Research, National Research
Centre, and Egypt. After the acclimatization period, the animals were divided into four groups (10 rats / group). Group (1): normal healthy animals served as negative control group, group (2): positive control group in which the rats were intraperitoneally administered with TAA (dissolved in 0.9% normal saline) in a dose of 200 mg / kg b.wt. Twice weekly according to Bruck et al[14] for 7 consecutive weeks for induction of liver fibrosis, group (3): *Punica granatum* control group in which the rats were treated orally with the methanolic extract of *Punica granatum* peel (dissolved in distilled water) in a dose of 400mg / kg b.wt. According to Rathod et al.[15] daily for 8 weeks and group (4): *Punica granatum* treated group in which the rats were treated orally with *Punica granatum* peel methanolic extract (dissolved in distilled water) in a dose of 400mg / kg b.wt. Daily for 8 weeks, following the administration of TAA 200mg/kg b.wt for seven weeks.

Body weight of rats was recorded at every week to monitor body weight change and to determine the doses for induction of liver fibrosis and treatment. At the end of the experimental period, animals were fasted 12 hours, then they were anaesthetized with diethyl ether and blood samples were collected from the retroorbital venous plexus in centrifuge tubes. One portion of each blood sample was collected on EDTA for separation of plasma and the other portion was collected in a tube free from anticoagulant for separation of serum. Both plasma and serum samples were separated by centrifugation at 1800 xg for 15 minutes at 4 °C using cooling centrifuge. Aliquots of plasma and serum were frozen and stored at -20°C for further biochemical analysis.

After blood collection, the animals were sacrificed by cervical decapitation, dissected and the whole liver of each rat was rapidly excised and thoroughly washed with isotonic saline. The whole liver of each animal in the different groups was divided into three portions; the first portion was snap-frozen directly in liquid nitrogen and stored at -80°C prior to RNA isolation for gene expression analysis, the second portion was homogenized in phosphate buffer (PH 7.4) to give 20% w/v homogenate[16]. This homogenate was centrifuged at 1700 xg and 4°C for 10 min , the supernatant was stored at -70°C until analysis. This supernatant (20 %) was used for the determination of hepatic reduced glutathione content. Whereas, the third portion of the liver was fixed in formal saline (10%) for histopathological examination.
METHODS

Biochemical analysis

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in serum were measured by kinetic method using Randox laboratories kits (UK) according to the methods recommended by the Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology [17]. Serum alkaline phosphatase (ALP) activity was estimated by colorimetric method[18] using kit from Quimica Clinica Aplicada (Amosta/Tarragona, Spain). Serum bilirubin level was determined by colorimetric method according to Scherlock [19] using Randox laboratories kit (UK). Hepatic reduced glutathione content was assayed by colorimetric method according to Beutler et al [20] using kit purchased from Bio-Diagnostic Co. (Egypt). Plasma fibrinogen was estimated by enzyme linked immunosorbent assay (ELISA) technique using fibrinogen ELISA kit purchased from Glory Science Co., Ltd (USA), according to the manufacturer’s instructions provided with fibrinogen assay kit. Serum hepatocyte growth factor (HGF) was estimated by enzyme linked immunosorbent assay (ELISA) technique using a kit purchased from Glory Science Co., Ltd (USA), according to the manufacturer’s instructions provided with HGF assay kit.

Gene expression analysis

100 mg of liver tissue was used for RNA extraction using PeqGold Trifast (Biotechnologie GmbH, USA) according to the manufacturer’s instructions. Primer sequence for β actin gene is: Forward, ccttcctgggccatgggtctct; Reverse, ggagcaatgtctgtcatcctc. Primer sequence for BCL-2 gene is: Forward, cctggtggacaacatcgcc; Reverse, aatcaaacagaggccgcatgc. Primer for NQO1 is: Forward, aacgtcattctctggccaattc, Reverse, gccaatgctgtacaccagttga. Qiagen on step RT PCR kit (Qiagen Inc, USA) was used for RNA reverse transcription and subsequent amplification. PCR reaction was performed separately for β actin and BCL-2 by adding 2 µg RNA to PCR mixture and making the reaction volume to 50 µl. PCR mixture contained 2 mM tris Cl, 10 mM KCl, (NH)2 SO4, 1.25 mM MgCl2, 0.1 mM dithiothreitol; PH 8.7, 0.4 mM dNTPs mixture, Qiagen one step RT PCR enzyme mix (contains OmniscriptTM Reverse transcriptase, SensiscriptTM Reverse transcriptase and Hot star TaqR DNA polymerase), and 0.6 µM of each specific primer. The reaction mixture was subjected to reverse transcription at 50°C for 30 minutes then to 35 cycles of PCR amplification as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. The PCR products were separated on 1.5% agarose gel and visualized by gel documentation.
system. The genes expression was semiquantified using LabImage analysis (LabImage2.7.0, Kapelan GmbH) software.

**Histopathological examination**

After fixation of liver tissues obtained from rats in the different studied groups in 10% formal saline for twenty four hours, washing was done in tap water. Then, serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by slidge microtome. The obtained tissue sections were collected on glass slides, deparaffinized, stained by hematoxylin & eosin stains. After that, examination was done through the light electric microscope [21].

**Statistical analysis**

Data were analyzed using version 13 of computer based Statistical Package for Social Sciences (SPSS). Results are expressed as means ± SD of three independent experiments. Statistical significance of difference was determined using analysis of variance (One way ANOVA). Further statistical analysis for post hoc comparisons was carried out using LSD test. A level of P <0.05 was defined as statistically significant.

**RESULTS**

The data presented in Table (1) showed that the induction of liver fibrosis in rats by thioacetamide significantly increases (P<0.05) the activity of aspartate aminotransferase (AST) in serum as compared to the negative control group (95.6 ± 2.7 vs 53.0± 3.2). *Punica granatum* control group showed insignificant change (P>0.05) in serum AST activity compared with the negative control group (51.0±3.9 vs 53.0±3.2). Significant decrease (P<0.05) in serum AST activity was recorded in *punica granatum* treated group as compared to the positive control (TAA group) (66.6± 2.7 vs 95.6±2.7). Significant increase (P<0.05) in serum alanine aminotransferase (ALT) activity was detected in the positive control group as compared to the negative control group (82.0 ± 1.5 vs 39 ±3.1). *Punica granatum* control group exhibited insignificant change(P>0.05) in serum ALT activity as compared to the negative control group (39.1±2.0 vs 39.0±3.1). Meanwhile, *Punica granatum* treated group showed significant decrease(P<0.05) in serum ALT activity as compared to the positive control (56.0±1.5 vs 82.0±1.5) (Table 1). In the same manner, significant increase(P<0.05) in serum alkaline phosphatase (ALP) activity was obtained in the
positive control group as compared to the negative control group (155.0 ± 2.6 vs 106.0±0.7). In contrast *Punica granatum* control group displayed significant decrease (P<0.05) in serum ALP activity compared with the negative control group (97.4± 1.2 vs 106.0± 0.7). *Punica granatum* treated group showed significant decrease (P<0.05) in serum ALP activity as compared to the positive control (114.6 ± 1.8 vs 155.0 ±2.6 ). Serum bilirubin level was significantly elevated(P<0.05) in TAA administered group (positive control group) as compared to the negative control group(0.78±0.008 vs 0.81±0.02) . *Punica granatum* control group revealed insignificant change (P>0.05) in serum bilirubin level compared with the negative control group (0.78±0.008 vs 0.81±0.02). Whereas, *Punica granatum* treated group showed significant decrease (P<0.05) in serum bilirubin level as compared to the positive control group (1.2±0.06 vs 2.06±0.03) (Table1).

**Table (1): Effect of treatment with *Punica granatum* peel methanolic extract on liver functions of rat model of liver fibrosis.**

<table>
<thead>
<tr>
<th></th>
<th>Negative control Group</th>
<th>Positive control group</th>
<th>Punica-granatum control group</th>
<th>Punica-granatum treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td>53.0±3.2</td>
<td>95.6 ± 2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.0 ± 3.9</td>
<td>66.6 ± 2.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>39.0 ±3.1</td>
<td>82.0 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.1 ± 2.00</td>
<td>56.0 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>106.0±0.7</td>
<td>155.0 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.4 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>114.6±1.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bilirubin(mg/dL)</td>
<td>0.81 ± 0.02</td>
<td>2.06±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.78 ± 0.008</td>
<td>1.2±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SD for 10 rats/group.

"a" significant difference as compared to the negative control at P<0.05

"b" significant difference as compared to the positive control at P<0.05

As shown in Table (2), hepatic reduced glutathione (GSH) content of rats with liver fibrosis (positive control group) was significantly decreased as compared to the negative control group (7.5±0.9 vs 12.8±0.8). *Punica granatum* control group showed insignificant change (P>0.05) in hepatic GSH content as compared to the negative control group (12.2±0.16 vs 12.8±0.8). Meanwhile, *Punica granatum* treated group displayed significant increase (P<0.05) in hepatic GSH content when compared with the positive control group (10.04±0.4 vs 7.5±0.9). Plasma fibrinogen level in rats with liver fibrosis (positive control group) revealed significant reduction (P<0.05) as compared to the negative control group (1808 ±6.2 vs 2116.6± 28). *Punica granatum* control group showed insignificant change (P>0.05) in plasma fibrinogen level compared with the negative control group (2110± 25 vs 2116.6± 28). However, *Punica granatum* treated group showed significant elevation (P< 0.05) in
plasma fibrinogen level as compared to the positive control group (2065±4.2 vs 1808±6.2). Also, the data illustrated in Table (2) revealed significant increase (P< 0.05) in serum hepatocyte growth factor (HGF) level in rat with liver fibrosis (positive control group) as compared to the negative control group (182.2±2.2 vs 104.6±1.7). *Punica granatum* control group showed insignificant change (P>0.05) in serum HGF level when compared with the negative control group (105.0±1.0 vs 104.6±1.7). Meanwhile *Punica granatum* treated group showed significant decrease (P<0.05) in serum HGF level as compared to the positive control group (128.1±1.2 vs 182.2±2.2).

**Table (2): Effect of treatment with *Punica granatum* peel methanolic extract on hepatic glutathione content, plasma fibrinogen and serum hepatocyte growth factor levels in rat model of liver fibrosis.**

<table>
<thead>
<tr>
<th></th>
<th>Negative control group</th>
<th>Positive control group</th>
<th><em>Punica granatum</em> control group</th>
<th><em>Punica granatum</em> treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced glutathione (mg/g.tissue)</td>
<td>12.8±0.8</td>
<td>7.5±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.24±0.16</td>
<td>10.04±0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FBG (µg/mL)</td>
<td>2116.6±28</td>
<td>1808±6.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2110±25</td>
<td>2065±4.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HGF (ng/L)</td>
<td>104.6±1.7</td>
<td>182.2±2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>105±1</td>
<td>128.1±1.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SD for 10 rats / group

FBG: Fibrinogen.

HGF: Hepatocyte growth factor

“a” significant difference as compared to the negative control at P<0.05

"b” significant difference as compared to the positive control at P<0.05

As shown in Table (3) and Fig (1), hepatic NQO1 gene expression level was significantly reduced (P<0.05) in the liver of rats with liver fibrosis (positive control group) compared with the negative control group (0.21±0.01 vs 0.87±0.01). Meanwhile, *Punica granatum* control group showed significant increase (P<0.05) in Hepatic NQO1 gene expression level as compared to the negative control group (0.98±0.01 vs 0.87±0.01). Similarly, significant increase (P<0.05) in hepatic NQO1 was detected in *Punica granatum* treated group when compared with the positive control group (0.67±0.03 vs 0.21±0.01). Hepatic BCL-2 gene expression level of the positive control showed significant reduction (P<0.05) as compared to the negative control (0.32±0.01 vs 0.69±0.01). Hepatic BCL-2 gene expression level in *Punica granatum* control group did not show significant change (P>0.05) as compared to the negative control group (0.63±0.12 vs 0.69±0.01). However, hepatic BCL-2 gene
expression level showed significant elevation (P<0.05) in *Punica granatum* treated group compared with the positive control group (0.49±0.02 vs 0.32±0.01).

**Table (3): Effect of treatment with *Punica granatum* peel methanolic extract on hepatic gene expression levels of NQO1 and BCL-2 in rat model of liver fibrosis.**

<table>
<thead>
<tr>
<th></th>
<th>Negative control group</th>
<th>Positive control group</th>
<th>Punica granatum control group</th>
<th>Punica granatum treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>NQO1</td>
<td>0.87±0.01</td>
<td>0.21±0.01</td>
<td>0.98 ±0.01</td>
<td>0.67±0.03</td>
</tr>
<tr>
<td>BCL-2</td>
<td>0.69±0.01</td>
<td>0.32±0.01</td>
<td>0.63±0.012</td>
<td>0.49±0.02</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SD for 10 rats/group.

“a” significant difference as compared to the negative control at P<0.05

“b” significant difference as compared to the positive control at P<0.05

Fig (1): RT–PCR product of hepatic NQO1, BCL-2 and β-actin genes expression in the negative control, positive control, *Punica granatum* control and *Punica granatum* treated rats. Lane 1 represents negative control, lane 2 represents positive control, lane 3 represents *Punica granatum* control, lane 4 represents *Punica granatum* treated rat and lane 5 represents DNA marker.

**Histopathological findings**

Histological examination of liver tissue sections of rats in the negative control group showed no histopathological alteration and the normal histological structure of the central vein and surrounding hepatocytes were noticed (Fig 2). Histological investigation of liver tissue sections of rats in the positive control group revealed multiple number of newly formed bile ductules with inflammatory cells infiltration and fibroblastic cells proliferation in the portal area with congestion in the central and portal veins. The hepatic parenchyma was divided into nodules by the proliferating fibroblasts and inflammatory cells infiltration (Fig 3). Histological examination of liver tissue sections of rats in *Punica granatum* control group
showed no histopathological alteration and the normal histological structure of the central vein and surrounding hepatocytes were observed (Fig 4). Histological investigation of liver tissue sections of rats in *Punica granatum* treated group revealed congestion in the central vein with perivascular oedema associated with inflammatory cells infiltration between the hepatocytes (Fig 5).

**Fig (2): Photomicrograph of liver tissue section of rats served as negative control showing normal histological structure**

**Fig (4): Photomicrograph of liver tissue section of rats in *Punica granatum* control group showing normal histological structure (H&Ex40).**

**Fig (3): Photomicrograph of liver tissue section of rats served as positive control showing multiple number of newly formed bile ducts (b) with inflammatory cells infiltration (m) and fibroblastic cell proliferation (f) in between as well as sever congestion in central (cv) and portal vein (pv) (H&Ex40).**

**Fig (5): Photomicrograph of liver tissue section of rats in *Punica granatum* treated group showing congestion of the central vein (CV) with perivascular oedema(H&Ex40).**

**DISCUSSION**

Liver diseases, a major cause of human mortality in developed and developing countries are heterogeneous concerning their aetiology, histology, clinical presentation and progression.
Despite the development of different therapeutic strategies for liver diseases, liver fibrosis, cirrhosis and its complications (liver failure, portal hypertension, and hepatocellular carcinoma) are not significantly reduced in many clinical situations\textsuperscript{[22]}. Thus, the high prevalence of liver diseases underscores the need for efficient and cost-effective treatment\textsuperscript{[23]}. Therefore, it is necessary to search for alternative therapy for the treatment of liver diseases to replace the currently used drugs of doubtful efficacy and safety.

Plants used in the traditional medicine for liver disorders are of great interest, as they may serve as potential sources for new therapeutic agents that could be applied in the management and prevention of hepatic injuries. Plants rich in different antioxidant derivatives have been reported to exhibit antihepatotoxic effects on experimental liver injury models induced by different types of hepatotoxicants\textsuperscript{[24-26]}.

This study was planned to investigate the therapeutic efficacy of \textit{Punica granatum} peel methanolic extract against TAA-induced liver fibrosis in rats. Thioacetamide (TAA) is a well-established agent to induce liver fibrosis in experimental animal's models\textsuperscript{[27]}.

When hepatic cell membrane is damaged, the enzymes ALT, AST and ALP which are normally located in the cytosol, leaked into circulation from hepatocytes\textsuperscript{[28]}. As a result, the activity of ALT, AST and ALP are increased in serum. Therefore the activities of ALT, AST and ALP are the most frequently used indicators for liver diseases. In this study, administration of TAA in rats caused significant increase in the activity of ALT, AST and ALP as well as bilirubin level in serum. These findings are in good agreement with those of earlier workers who reported similar biochemical changes\textsuperscript{[29]} as thioacetamide causes oxidative stress and enhances free radical-mediated damage to proteins, lipids and deoxyribonucleic acid (DNA)\textsuperscript{[30-33]}. Such oxidative stress was indicated by the occurrence of hepatocellular injury leading to cell necrosis and discharge of the contents of the hepatocytes into the blood stream. It has been reported that serum ALT and AST activities are very sensitive indicators for necrotic lesions within the liver because their ease liberation from the hepatocyte cytoplasm into the blood stream\textsuperscript{[28]} as a result of membrane lipid peroxidation\textsuperscript{[34]}. TAA is known to cause changes in cell membrane permeability by its metabolite thioacetamide-S-oxide\textsuperscript{[35]} and the elevated serum enzymes are indicative of cellular leakage and loss of functional integrity of the cell membrane of the liver\textsuperscript{[36]}. The elevated serum level of bilirubin in TAA administered group in the current study is in agreement with the previous results of El-Kott and Owayss\textsuperscript{[37]}. The increased bilirubin level
in serum indicates the diffused harm to the liver with more and more liver tissue damage [38]. Thus, the elevated serum values of each of ALP and bilirubin in TAA administered rats clearly indicates the liver injury in concomitant with liver dysfunction due to TAA intoxication. As serum ALP activity and bilirubin level are almost related to the function of hepatic cells [25].

Interestingly, treatment with Punica granatum peel methanolic extract for 8 weeks effectively ameliorated the significant elevation in serum ALT, AST, ALP activities and bilirubin level in TAA administered rats. These findings are in agreement with those of Abdel-Rahman et al. [39] who found that administration of pomegranate peel extract significantly reduced the damaging impact of CCl4 on the liver. The effect of Punica granatum on liver enzymes could be attributed to the antioxidant activity of its active constituents. Pomegranate juice, peel, seeds – all have a potent antioxidant activity due to their active compounds that are electron donors, which can react with free radicals to convert them to more stable products and terminate radical chain reaction [40, 41]. The inhibitory effect of Punica granatum on lipid peroxidation in rat liver microsomes is related to the ability of phenolic and flavonoid compounds present in its extract to inhibit hepatic oxidative enzymes (cytochrome P450 system) [42, 43]. Lipid peroxidation product, MDA, levels has been found to be lowered significantly upon treatment of intoxicated rats with the ethanolic extract of Punica granatum peel, compared with untreated CCl4-intoxicated rats [44]. Moreover, it has been found that the increase tissue MDA levels due to bile duct ligation (BDL) are also reduced back to control levels by treatment with pomegranate peel extract (PPE) in rats [45]. Thus, the present findings indicate that Punica granatum peel extract preserves the structural integrity of the hepatocellular membrane and liver cell architecture as confirmed by our histopathological results. Therefore, it could prevent the leakage of liver enzymes into serum and restore the liver functions.

Fibrinogen is a glycoprotein of molecular weight approximately 340,000 daltons, present in the plasma at a concentration in the range of 2–4 g/L. It is synthesized in the liver (1.7–5 g/day), and by the megakaryocytes [46]. In this study, plasma fibrinogen levels showed significant decrease in TAA-administered group as compared to the negative control group. This finding is in agreement with Chang et al [47]. Fibrinogen is one of the major acute phase proteins synthesized by the liver and its concentration is often used as a surrogate for systemic inflammation [48]. Aster [49] reported that low level of fibrinogen among other
coagulation proteins in fibrosis is classically ascribed to insufficient hepatic synthesis. In addition, the decreased fibrinogen level in liver fibrosis may occur due to the increase of fibrinogen degradating products. Plasma fibrinogen level was significantly increased in *Punica granatum* treated rats as compared to the positive control ones. Kaur et al. [10] demonstrated that alcoholic extract of *Punica granatum* flower possesses potent free radical scavenging property, antioxidant capacity and hepatoprotective activity. Similarly, the present finding further indicated that *Punica granatum* peel methanolic extract attenuates the liver injury induced by TAA and maintains the proper functions of hepatic cells to produce fibrinogen as a result of the antioxidant potential of its active principles.

It is well known that the toxicity of TAA results from its bioactivation in the liver to its reactive metabolites causing the production of ROS responsible for oxidative stress [50]. This is followed by glutathione depletion, reduction of SH-thiol groups and oxidation of cell macromolecules, including lipids [51]. The present study showed significant depletion in hepatic glutathione content in TAA administered rats. This finding coincides with that of Sanz et al. [52] and Diez-fernandez et al. [53]. In tissues, glutathione occurs in a reduced (GSH) and oxidized form (GSSG) and more than 99% of the total glutathione occurs as GSH [54, 55]. Glutathione plays a key role in detoxification of ROS and reactive electrophilic compounds [56, 57]. TAA is known to induce hepatocyte damage following its metabolism to thioacetamide sulphene and sulphone, *via* a critical pathway involving cytochrome P450-mediated biotransformation [50]. These metabolites are highly reactive and thus lead to the denaturation of cellular biomolecules such as lipids, resulting in lipid peroxidation [58]. The mechanisms that contribute to the occurrence of lipid peroxidation do not only include oxygen free radical generation, but also include alterations in the cellular antioxidant defense system with a decline in the intracellular free radical scavengers [59]. The underlying mechanism for the decline in hepatic reduced glutathione content by TAA in our study may be related to the inhibition of its regenerating enzyme, glutathione reductase (GSH-Rx), by TAA administration [60]. Reduced glutathione (GSH) is regenerated from oxidized glutathione (GSSG) and NADPH in a reaction catalyzed by GSH-Rx. NADPH, in turn, is generated *via* the hexose monophosphate shunt by a reaction catalyzed by glucose 6 phosphate dehydrogenase (G6PD) [61]. Therefore, the deficiency of GSH content in the liver may be also attributed, in part, to a deficiency in G6PDH which is considered a housekeeping enzyme that catalyses the first step in the pentose phosphate pathway to produce NADPH, which is necessary for the reduction of GSSG to GSH [62]. The current study revealed
significant elevation in hepatic reduced glutathione content in *Punica granatum* treated group. Some studies demonstrated the presence of flavonoids, steroids, terpenoids and tannins in *Punica granatum*. Flavonoids have been found to reduce xenobiotic-induced hepatotoxicity in animals and counteract the damaging effects of oxidative stress, cooperating with natural systems like glutathione and other endogenous protective enzymes \[63,64\]. In a series of experiments, it was demonstrated that relatively low concentrations of flavonoids stimulate the transcription of a critical gene for GSH synthesis in the cells \[65\].

NADPH quinone oxidoreductase 1 (NQO1) is the major phase-II detoxification enzyme controlled by *Nrf2* which is an important transcription factor that regulates antioxidative stress reactions \[66\]. In this study, TAA administered group showed significant reduction in hepatic NQO1 gene expression level as compared to the negative control group. Demirel et al. \[67\] stated that TAA administration in rats lowered *Nrf2* gene expression in the liver. And hence, the significant reduction of NQO1 gene expression in TAA-administered rats could be attributed to the lowering effect of TAA on *Nrf2* gene expression.

Interestingly, the significant elevation in hepatic NQO1 gene expression was detected in *Punica granatum* treated group. This elevation indicated the restoration of hepatic NQO1 gene expression level in the treated rats. This finding could be explained by the ability of *Punica granatum* active ingredients to reverse the deteriorated antioxidant enzymes activity in the liver tissue of rats intoxicated with TAA through scavenging free radicals, decreasing lipid peroxidation, attenuating the liver susceptibility to oxidative stress. Thus, the extract of *Punica granatum* with its active components (flavonoids) could improve cellular membrane and organ functioning more profoundly and bring NQO1 gene expression levels towards the control values \[68\].

Hepatocyte growth factor (HGF), also known as *SF* (scatter factor) is a pleiotropic cytokine involved in many complex biological processes, from embryogenesis and tissue regeneration to tumour growth, metastasis and angiogenesis \[69\]. Serum hepatocyte growth factor (HGF) was significantly increased in rats administered TAA as compared to the negative control ones. HGF play an important role in liver regeneration as an endocrine or paracrine factor. In the liver, HGF is synthesized by non parenchymal cells \[70\] and targets both parenchymal hepatocytes and bile duct epithelial cells. The signal-transducing receptor for HGF is the c-met protooncogene product of transmembrane tyrosine kinase \[71\]. Matsumoto and Nakamura
reported that HGF mRNA and HGF activity are increased in the liver of rats after various liver injuries. Also, HGF level is increased markedly in mouse liver after various liver injuries such as hepatitis, ischemia, physical crush and partial hepatectomy\textsuperscript{72} HGF promotes hepatic survival by stimulating liver regeneration and providing hepatoprotection in various models of liver injury. It has been proven that HGF, transforming growth factor-\(\alpha\) (TGF-\(\alpha\) ) and epidermal growth factor (EGF) are the main growth factors secreted after hepatic injury. HGF is the most potent mitogen for mature hepatocytes and acts as a hepatotrophic factor.

The increased serum HGF level was restored to near normal levels in \textit{Punica granatum} treated group as compared to the positive control group. This finding is in agreement with that of Bassiony et al.\textsuperscript{73} who reported that \textit{Punica granatum} significantly reduce HGF levels in rats. This effect may be due to \textit{Punica granatum} flavonoid content as Huang et al.\textsuperscript{72} reported that flavonoids produce significant reduction in HGF levels after liver intoxication. Polyphenols have been found to inhibit receptor kinases such as EGFR, HER-2, c-met, platelet-derived growth factor receptor, insulin-like growth factor receptor, vascular endothelial growth factor receptor, and downstream kinases including Erk1/2, STAT3, and P13K\textsuperscript{74,75}. This may be a mechanism underlying the effect of \textit{Punica granatum} methanol extract to restore the serum level of HGF towards the normal value.

\textit{BCL-2} gene, is the founding member of the \textit{BCL-2} family of regulator proteins that regulate cell death (apoptosis)\textsuperscript{76}. Upregulated apoptosis of hepatocytes is increasingly viewed as a nexus between liver injury and fibrosis. In this study TAA administration produced, significant decrease in \textit{BCL-2} gene expression level in the liver. Cytotoxic drugs and cellular stress activate the intrinsic mitochondrial apoptotic pathway\textsuperscript{77} so that the inhibitors of \textit{BCL-2} activate caspase-3 initiating apoptosis, whereas inducers of \textit{BCL-2} induce necrosis of cells\textsuperscript{78}. TAA has been reported to cause upregulation of Bax protein and downregulation of the antiapoptotic protein \textit{BCL-2} and its translocation into the mitochondria, causing apoptosis\textsuperscript{79}. This finding is in accordance with results of the present study.

\textit{Punica granatum} treated group showed significant elevation in Bcl-2 gene expression level in the liver indicating the ameliorative effect of this treatment on TAA-induced apoptosis. Intracellular ROS loading may directly entail the depletion of intrinsic antioxidant potentials and the activation of the transduction pathways leading to apoptosis\textsuperscript{80}. Choi et al.\textsuperscript{81} stated that polyphenolic flavonoids, the active constituents of \textit{Punica granatum}, may differentially prevent Cu\textsuperscript{2+}-oxidized LDL-induced apoptosis and promote cell survival.
In the histopathological investigations of this study, numerous macronodules were seen on the surface of fibrotic livers of rats given TAA for 6 consecutive weeks (positive control). Moreover, the liver tissue section showed portal fibrosis and expansion of connective tissue (portal and peripheral fibrosis). Furthermore, multiple numbers of newly formed bile ductules with inflammatory cells infiltration and fibroblastic cells proliferation were detected in the portal area accompanied with congestion in the central and portal veins. The hepatic parenchyma was divided into nodules by the proliferating fibroblasts and inflammatory cells infiltration. Similar findings have been demonstrated by Nozu et al. [27] who found that TAA administration produces portal fibrosis consisting of expansion of the connective tissue of the portal area. Also chronic poisoning with TAA by intraperitoneal injection induced changes in the periportal collagen deposit[82]. Moreover, progressive changes in the liver, such as cellular necrosis and regeneration as well as hepatic fibrosis with the formation of pseudo-lobules due to TAA intoxication have been reported by Zsigmond et al. [83].

In our experiment, treatment with *Punica granatum* peel extract daily for 8 weeks after 6 weeks of TAA administration ameliorated liver tissue damage to great extent as indicated by the presence of perivascular oedema with the congestion of the central vein only. This effect could be attributed to the enhancing effect of *Punica granatum* on tissue regeneration[84].

**CONCLUSION**

the current study provided a clear evidence on the antifibrotic potential of *Punica granatum* peel methanolic extract in the experimental model of liver fibrosis. This effect could be attributed to the antioxidant as well as the antiapoptotic properties of this extract. Thus, the present study represents a good therapeutic approache for intervention against liver fibrosis.

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