Silymarin mitigates diclofenac-induced liver toxicity through inhibition of inflammation and oxidative stress in male rats

Pantea Ramezannezhad¹, Ali Nouri*¹, Esfandiar Heidarian²

¹Medical Plants Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran
²Clinical Biochemistry Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran

Introduction
Drug induced liver toxicity is one of the main worries for pharmaceutical industry and physicians (1). Non-steroidal anti-inflammatory drugs have anti-inflammatory and analgesic properties that are widely prescribed and some of them cause liver toxicity (2,3). Diclofenac (DIC) belongs to Non-steroidal anti-inflammatory drug family and it is one of the compounds derived from phenyl acetic acid but liver toxicity is one of the main concerns for this drug (4). The mechanism of liver toxicity induced by DIC has been partly associated to mitochondrial damage (5,6), disruption of antioxidant defense system (7), change in the integrity of covalent protein by reactive metabolites (8), and the mechanisms mediated by the immune system (9). DIC is listed under FDA black box warning for cardiovascular toxicity and liver toxicity with mitochondrial liabilities (10). Therefore, any therapeutic agent which can suppress any of the pathological pathways activated by DIC might be used to prevent or inverse its toxic action.

Silymarin is a flavonoid compound which is derivate from Silybum marianum seeds. It is a combination of silydianine, silychristine, silibinin and isosilybinin (11). Previous studies have shown that silymarin has

The present study showed that silymarin mitigates DIC-induced liver toxicity through inhibition of inflammation and oxidative stress in male rats. After clinical trials, silymarin may be used with DIC as a complementary drug for decreasing the toxicity of DIC.

antioxidant and anti-inflammatory properties (12,13). This research was aimed to assess the noxious effect of DIC intoxication on the liver through serum biochemical markers, oxidative stress, gene expression of tumor necrosis factor-α (TNF-α), histological alterations and protective role of silymarin on DIC intoxication in rats.

Materials and Methods

Chemicals
2-thiobarbituric acid, sodium acetate, and H₂O₂ were prepared from Merck company (Darmstadt, Germany). Silymarin, 5,5-dithiobis-2-nitrobenzoic acid, griess reagent, GSH, nitro blue tetrazolium and TPTZ (2,4,6-tripyridyl-s-triazine) were purchased from Sigma (St. Louis, MO). SYBR Green Real Time-PCR Master Mix was provided from Qiagen Co. (Dusseldorf, Germany). Total bilirubin, alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were evaluated using kits of Pars Azmun co (Tehran, Iran). All other chemicals which employed in this study were of analytical grade.

Animals
Forty male Wistar rats weighting around 220 g (6–8 week old) were applied for this research. The experimental rats were obtained from Jondishapour University (Ahwaz, Iran). The rats were kept under 25°C ambient temperature, 50% relative humidity and a dark– light cycle (12 hours) and were allowed free access to water and standard diet. All methods were confirmed by the Ethics Committee of Shahrekord University of Medical Sciences Ethics Committee, Shahrekord, Iran (Ethic number IR.SKUMS.REC.94.146).

Experimental design
Randomly, 40 male Wistar rats were assigned into five groups each comprising of eight animals and treated for 5 days. Group 1 (control group) was treated with normal saline (1 mL/kg, i.p.). Group 2 received DIC only (50 mg/kg bw, i.p.) (14). Group 3 received silymarin only (200 mg/kg). Group 4, 5 received DIC (50 mg/kg bw, i.p.) (14). Group 3 received silymarin only (200 mg/kg). Group 1 (control group) was treated with normal saline (1 mL/kg, i.p.). Group 2 received DIC only (50 mg/kg bw, i.p.) (14). Group 3 received silymarin only (200 mg/kg). Group 4 received DIC (50 mg/kg bw, i.p.), silymarin (100, 200 mg/kg bw, i.p.) plus silymarin (100, 200 mg/kg bw, p.o., respectively) (15-17). At the end of our study, fasted rats were killed under mild chloroform anesthesia. Whole blood specimens were gathered by cardiac puncture procedure to obtain serum and plasma. Also, liver tissue was removed for histological studies. At the end of our study, fasted rats were killed under mild chloroform anesthesia. Whole blood specimens were gathered by cardiac puncture procedure to obtain serum and plasma. Also, liver tissue was removed for histological studies.

Biochemical analysis
Serum biochemical markers such as total bilirubin, ALP, AST and ALT contents were measured by an enzymatic method (Pars Azmun kit, Pars Azmun Co., Tehran, Iran) using autoanalyzer (BT 3000, Biotecnica, Cergy Pontoise Cedex, France).

Determination of ferric reducing/antioxidant power (FRAP)
Ferric reducing/antioxidant power was determined by Heidarian and Soofiniya procedure (18). Briefly, plasma was mixed with FRAP reagent (composed of: 2.5 ml acetate buffer, 2.5 ml TPTZ solution and 2.5 ml FeCl3) then 10 minutes incubated at 37 centigrade degree and optical density of blue color was measured at 593 nm. FeSO4 was used as a standard of FRAP assay at a concentration range between 100 and 1000 μM. The results are reported as μmol/L.

Determination of nitrite content
Content of nitrite was evaluated using a griess reagent (19). Briefly, liver supernatant was blended with griess reagent (composed of: 0.1% N-(1-naphthyl) ethylene diamine, 1.0% sulfanilamide and 2.5% orthophosphoric acid) then, 30 minutes incubated at 37 centigrade degree and light absorbance was measured at 548 nm. Content of nitrite was assessed using standard curve of sodium nitrite and was reported as μM/mg tissue.

Determination of lipid peroxidation
MDA contents in rat liver and serum were determined by the TBA reactive substance test as described previously (18). Briefly, plasma or supernatant was mixed with sodium dodecyl sulfate (8.1%) and TBA/buffer (composed of: of 0.53% thiobarbituric acid in 20% acetic acid as adjusted to pH 3.5 with NaOH) then, 60 minutes incubated at 95 centigrade degree. The reaction was stopped by placing tubes on ice followed by centrifugation at 4000 rpm for 10 minutes. The optical density of pink color was measured at 532 nm. 1, 1, 3, 3-tetraethoxypropane was used as a standard of MDA assay. The measurements were done in duplicates and the results were expressed in μmol/L.

Determination of intracellular GSH levels
Intracellular GSH contents were determined using of Ellman method (20). In this procedure 5,5-dithiobis-2-nitrobenzoic acid reacts with GSH and the optical density has a peak value at 410 nm. Data were reported as μmol/g tissue.

Tissue GPx activity
The activity of GPx was assayed by determining the reduction of GSH level after adding the sample in the presence of H₂O₂ and NaN3 (21).

Determination of tissue CAT and SOD activities
The liver CAT activity of experimental groups was measured as described previously by Heidarian et al (22). Briefly, H₂O₂ solution (composed of: 10 mmol H₂O₂, ...
Effects of silymarin on diclofenac-induced liver toxicity

in 50 mM phosphate buffer, pH 7.4), and homogenate was pipetted into a cuvette. The reduction of H2O2 was followed at a wave length of 240 nm for 2 minutes. The activity of liver SOD was evaluated using the inhibition of the NBT photo chemical reaction at 560 nm (23). All total protein samples were assessed using the method of Bradford (24).

Determination of serum TNF-α
Serum TNF-α was assessed using the ELISA assay kit (BT-Laboratory, China) according to the procedure provided by the manufacturer.

Determination of TNF-α gene expression
The mRNA was extracted using the BIOZOL kit reagent (Bioer, China) according to the instructions. Quality and quantity of total RNA were measured by absorbance of 260/280 nm using a spectrophotometer by the Nanodrop2000 (Thermo USA). cDNA measurement was done by the PrimeScript™ reagent kit (Takara Bio Inc. Japan) and in accordance with the instructions. Then, cDNA amplified according to RT-qPCR and using SYBR® Green PCR Master Mixture was used in the presence of specific primers for TNF-α (forward: 5’-CTGGCGTTTCATCCGGTCTC-3’, reverse: 5’-GGCTCTGAGTAGAATCAGTA-3’) and β-actin (forward: 5’-CTTCTACATGAGCCGTGTCG-3’ , reverse: 5’-GGAACATGATCTTTGATCTGGGT-3’). Primers after design with Oligo 7.0 software was approved using Blast-Nucleotide (NCBI). PCR was performed in 40 cycles (including secondary denaturation at 15 seconds at 95°C, 20 seconds at 60°C for annealing, 25 seconds for 72°C for extension). β-actin gene was used as an internal control gene to control the expression of mRNA. Also, ΔΔCT method was used for the analysis of gene expression.

Histopathological study
A segment of each liver was fixed in 10% formalin solution. All samples were embedded in paraffin and 5 µm thickness sections were prepared. Then, the slices stained with haematoxylin–eosin (H & E) for histopathological observation (25,26).

Statistical analysis
One-way analysis of variance (ANOVA) was used for data analysis using SPSS software (Statistical Package for the Social Sciences, version 20.0, SPSS Inc, Chicago, IL). All data were expressed as mean ± SD. Also, ΔΔCT method was used for the analysis of TNF-α gene expression. Mean values of the groups were compared using the Tukey post hoc test. P<0.05 was statistically considered significant.

Results
Effects of silymarin on serum ALT, AST, ALP and total bilirubin
Table 1 shows the effects of DIC and silymarin on total bilirubin, ALP, AST and ALT in serum. Injection of DIC in DIC-only treated group led to a noticeable elevation (P<0.05) in serum ALT, AST, ALP and total bilirubin relative to control animals (Table 1). However, administration of silymarin led to noticeable decline (P<0.05) in ALT, AST, ALP and total bilirubin in animals exposed to DIC.

Effects of silymarin on plasma FRAP, Nitrite content and MDA levels
The effects of silymarin on MDA levels, Nitrite content and plasma FRAP are shown in Table 2. In the DIC-only treated group, injection of DIC resulted in a remarkable decrease (P<0.05) in plasma FRAP and a noticeable increase in nitrite content, liver MDA and serum MDA contents relative to control animals. Treatment with silymarin caused a noticeable elevation (P<0.05) in plasma antioxidant capacity (FRAP) and a noticeable decrease (P<0.05) in nitrite content, liver MDA and serum MDA contents in animals exposed to DIC.

Effects of silymarin on CAT activity, SOD activity, GPx activity and GSH level
Table 3 shows that injection of DIC led to a noticeable reduction (P<0.05) in hepatic CAT and SOD activities in DIC-only treated group relative to the control animals. A considerable elevation (P<0.05) in hepatic SOD and CAT activities was observed after treatment with silymarin in

Table 1. Effects of silymarin on serum ALT, AST, ALP and total bilirubin in the experimental groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>26.13 ± 5.73</td>
<td>81.02 ± 7.95</td>
<td>25.32 ± 6.12</td>
<td>38.09 ± 4.53</td>
<td>28.26 ± 3.06</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>50.66 ± 2.28</td>
<td>110.4 ± 10.42</td>
<td>49.35 ± 3.12</td>
<td>62.12 ± 3.62</td>
<td>52.76 ± 2.85</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>139.4 ± 4.75</td>
<td>428.1 ± 7.25</td>
<td>140.2 ± 4.38</td>
<td>252.32 ± 5.85</td>
<td>146.21 ± 5.38</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.85 ± 0.02</td>
<td>2.4 ± 0.05</td>
<td>0.83 ± 0.03</td>
<td>1.02 ± 0.04</td>
<td>0.87 ± 0.03</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM (n=8) and analyzed by one-way ANOVA followed by Tukey post hoc test. Group 1: control animals; Group 2: DIC-only treated animals; Group 3: silymarin-only treated animals (200 mg/kg); Groups 4 and 5: the animals were treated by DIC plus silymarin at doses of 100 mg/kg and 200 mg/kg, respectively. *P<0.05 versus control animals (group 1), †P<0.05 versus DIC-only treated animals (group 2), ‡P<0.05 versus silymarin-only treated animals (group 3), §P<0.05 versus animals treated by DIC plus silymarin at dose of 100 mg/kg (group 4).

http://www.herbmedpharmacol.com
animals exposed to DIC. Table 3 also shows a remarkable decrease (P<0.05) in liver GPx activity in the DIC-only treated group compared to the control animals. However, administration of silymarin at doses of 100 and 200 mg/kg remarkably elevated liver GPx activity in DIC exposed rats. Also injection of DIC in DIC-only treated group led to a noticeable reduction (P<0.05) in liver GSH relative to the control group (Table 3). Nevertheless, treatment with silymarin at doses of 100 and 200 mg/kg let to remarkable elevation (P<0.05) in liver GSH in rats exposed to DIC.

The effects of silymarin on serum and gene expression of TNF-α
Figure 1 shows the effects of silymarin on level of serum TNF-α and TNF-α gene expression. DIC injection in DIC-only treated group led to a remarkable increase (P<0.05) in serum TNF-α level and TNF-α gene expression compared to the control group. Treatment with silymarin led to a noticeable reduction (P<0.05) in level of serum TNF-α and TNF-α gene expression in DIC exposed animals. A remarkable change (P<0.05) was seen in gene expression and serum level TNF-α in the group treated with silymarin at dose of 100 mg/kg in comparison with the group treated with silymarin at dose of 200 mg/kg.

Histopathological findings
The histopathological results are demonstrated in Figure 2. The microscopic examination demonstrated normal structures of liver tissue from a control rat in the control group (Figure 2A). Injection of DIC in DIC-only treated

Table 2. Effects of silymarin on FRAP, Nitrite content and MDA levels in the experimental groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum FRAP (μM)</td>
<td>526.27 ± 31.22</td>
<td>374.26 ± 27.41</td>
<td>618.67 ± 50.21</td>
<td>498 ± 37.64</td>
<td>612.25 ± 48.66</td>
</tr>
<tr>
<td>Nitrite content (μM/mg tissue)</td>
<td>5.63 ± 0.21</td>
<td>13.32 ± 0.89a</td>
<td>5.51 ± 0.32</td>
<td>7.48 ± 0.38abc</td>
<td>5.56 ± 0.28abc</td>
</tr>
<tr>
<td>Serum MDA (nmol/L)</td>
<td>8.43 ± 2.52</td>
<td>19.68 ± 3.18a</td>
<td>7.96 ± 2.39</td>
<td>13.65 ± 2.15abc</td>
<td>8.17 ± 1.87abc</td>
</tr>
<tr>
<td>Liver MDA (nmol/mg protein)</td>
<td>1.91 ± 0.71</td>
<td>5.51 ± 1.15a</td>
<td>1.80 ± 0.67</td>
<td>3.56 ± 0.76abc</td>
<td>1.87 ± 0.58abc</td>
</tr>
</tbody>
</table>

FRAP (ferric reducing/antioxidant power), MDA (malondialdehyde).
Data are expressed as mean ± SEM (n=8) and analyzed by one-way ANOVA followed by Tukey post hoc test. Group 1: control animals; Group 2: DIC-only treated animals; Group3: silymarin-only treated animals (200 mg/kg); Groups 4 and 5: the animals were treated by DIC plus silymarin at doses of 100 mg/kg and 200 mg/kg, respectively. a P<0.05 versus control animals (group 1), b P<0.05 versus DIC-only treated animals (group 2), c P<0.05 versus silymarin-only treated animals (group 3), d P<0.05 versus animals treated by DIC plus silymarin at dose of 100 mg/kg (group 4).

Table 3. Effects of silymarin on CAT activity, GPx activity and GSH level in the experimental groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT (U/mg protein)</td>
<td>165.32 ± 14.32</td>
<td>55.27 ± 6.12a</td>
<td>169.18 ± 13.21b</td>
<td>112.87 ± 9.76abc</td>
<td>167 ± 15.13b</td>
</tr>
<tr>
<td>SOD (U/mg protein)</td>
<td>46.32 ± 4.12</td>
<td>27.61 ± 2.89a</td>
<td>48.96 ± 5.11a</td>
<td>37.21 ± 3.13abc</td>
<td>47.43 ± 3.98abc</td>
</tr>
<tr>
<td>GPx (U/mg protein)</td>
<td>26.34 ± 0.89</td>
<td>20.12 ± 0.76a</td>
<td>26.94 ± 0.98a</td>
<td>23.12 ± 0.61abc</td>
<td>25.86 ± 1.07abc</td>
</tr>
<tr>
<td>GSH (μmol/g tissue)</td>
<td>12.89 ± 0.31</td>
<td>5.01 ± 0.09b</td>
<td>13.11 ± 0.19b</td>
<td>8.86 ± 0.09b</td>
<td>13.02 ± 0.17b</td>
</tr>
</tbody>
</table>

CAT (catalase), SOD (superoxide dismutase), GPx (glutathione peroxidase), GSH (reduced form of glutathione). Data are expressed as mean ± SEM (n=8) and analyzed by one-way ANOVA followed by Tukey post hoc test. Group 1: control animals; Group 2: DIC-only treated animals; Group3: silymarin-only treated animals (200 mg/kg); Groups 4 and 5: the animals were treated by DIC plus silymarin at doses of 100 mg/kg and 200 mg/kg, respectively. a P<0.05 versus control animals (group 1), b P<0.05 versus DIC-only treated animals (group 2), c P<0.05 versus silymarin-only treated animals (group 3), d P<0.05 versus animals treated by DIC plus silymarin at dose of 100 mg/kg (group 4).
group demonstrated mononuclear cells infiltration and histological changes compared to the control group that reflected liver toxicity (Figure 2B). Treatment with silymarin led to a noticeable reduction in inflammatory cell infiltration in groups exposed to DIC (Figure 2D&E). The decrease of inflammatory cells in treated group with silymarin at dose of 200 mg/kg was more than treated group with silymarin at dose of 100 mg/kg.

Discussion
Our findings showed that DIC at 50 mg/kg induced liver toxicity in male rats and silymarin prevented its hepatotoxic effects. The release of specific enzymes such as AST, ALT, and ALP into circulatory system is one of the most sensitive signs of liver damage. In our study noticeable elevation was seen in the contents of total bilirubin, AST, ALT and ALP in DIC-only treated group (Table 1) compared to the control animals which approved the liver toxicity potential of DIC. These results are in agreement with prior reports (5, 14, 27). In this study treatment with silymarin at doses of 100 and 200 mg/kg reversed changes induced by DIC in the contents of ALT and AST. This indicates that silymarin protected the structural integrity of liver cell membranes and eventually inhibited the leakage of these enzymes into circulatory system. The elevation in the contents of total bilirubin and ALP observed in the DIC-only treated animals is a marker of cholestasis in obstructive jaundice (28). Cysteinyl leukotrienes play an important role in inflammatory responses and the escalation of cholestasis in obstructive jaundice (28). In this research, reduction in levels of total bilirubin and ALP in silymarin treated groups may be due to its anti-inflammatory and antioxidant properties. There seems to be difference in the effect of silymarin at doses of 100 and 200 mg/kg on these markers. Therefore, it can be said that the effect of silymarin on these biochemical markers is dose sensitive.

Histopathological studies of DIC-only treated group depicted increased lymphocyte infiltration and apoptotic changes, which show liver toxicity induced by DIC in rats according to prior investigations (14, 27). Remarkable recovery towards normal liver histopathology in silymarin administrated groups further supported the protective effect of silymarin on DIC-induced hepatotoxicity.

MDA is a reliable marker for lipid peroxidation (LPO). Increased LPO by DIC as one of the signs for oxidative stress published previously by various studies (14, 27, 29). In this research, DIC-induced liver toxicity led to a noticeable elevation in the content of MDA in serum and hepatic tissue relative to the control animals that is in line with the prior published investigations (5, 30, 31). On the other hand, administration of silymarin not only increased the FRAP content in plasma but also decreased the MDA level in the serum and hepatic tissue in rats exposed to DIC. The elevated FRAP content and decreased LPO level in silymarin treated groups might be due to direct increase in GSH content or due to its free radical scavenging property.

Increase in level of nitrite in DIC-only treated rats indicated the effect of NO in liver toxicity induced by DIC. Our results are in line with the prior studies that have shown NO creates important role in chemical toxicity (32, 33). Remarkable reduction of nitrite content in silymarin treated groups suggested that this antioxidant offered liver protection in DIC exposed rats by reduce the NO content and nitrosative stress.

Intracellular glutathione (GSH), the principal endogenous antioxidant, creates a critical role in defense of cell on damage induced by oxidative stress. Both in vivo and in vitro studies have shown the effect of GSH depletion in oxidative stress induced by DIC in various model systems (27, 30, 34). In our research, DIC-induced liver toxicity led to a noticeable reduction in the content of GSH in liver tissues relative to the control animals. On the other hand, a noticeable recovery of GSH level was observed in silymarin administrated groups. GSH recovery may be due to the ability of silymarin to reduce oxidative stress or to increase the GSH content directly.

In order to survey the role of silymarin on metabolism of
GSH, our study assessed its role on GPx as GSH metabolizing enzyme. GPx simplifies peroxides neutralization via react with GSH and leading to increased contents of GSSG (oxidized glutathione), that is then reduced by glutathione reductase return to the GSH thereby maintaining the antioxidant content. DIC-induced changes in activity of GPx have been indicated previously (14,30). In our study, DIC-induced increase in GPx activity (Table 3) may be due to the defense mechanism against elevated LPO and noticeable reduction in GPx activity in the treated groups with silymarin can be due to the decrease levels of LPO or oxidative stress.

Previous investigations have indicated that injection of DIC causes a remarkable reduction in CAT and SOD in animals (5,14,30). In our investigation, injection of DIC caused the reduction in activities of CAT and SOD. On the other hand, treatment with silymarin remarkably restored these alterations of enzymatic activities in animals. The decrease in SOD and CAT activities in silymarin treated rats could be due to antioxidant properties of silymarin.

TNF-α is a cytokine which releases in the inflammatory conditions and migration of macrophages. TNF-α leads to oxidative stress which in turn increases the liver cells damage (35). Previous researches have shown that injection of DIC led to remarkable elevate in serum level of TNF-α and its gene expression in hepatic tissue (5,14,30). The result of our study showed that liver TNF-α gene expression and serum TNF-α content elevated remarkably in the DIC-only treated group relative to the control animals. In our investigation, liver TNF-α gene expression and serum TNF-α level significantly decreased in the silymarin-treated rats. Thus, the decrease in TNF-α serum content and expression of TNF-α is another obvious, at least partly, which confirms the liver protective role of silymarin due to its antioxidant properties.

**Conclusion**
Our study indicates that silymarin has protective effect against DIC-induced liver toxicity in rats. Also, silymarin as a natural component due to its anti-inflammatory and antioxidant properties may lead to improvement in complications of DIC consumption such as liver toxicity.

**Acknowledgments**
We would like to express our gratitude to those who have helped us in Clinical Biochemistry Research Center of Shahrekord University of Medical Sciences.

**Authors’ contributions**
Designing the study, data analysis and preparation of the manuscript was done by AN and PR. Authors read and approved the final manuscript.

**Conflict of interests**
The authors declared no competing interests.

---

**Ethical considerations**
The study was approved by the Ethical Committee of Shahrekord University of Medical Sciences, Shahrekord, Iran (Ethic number: IR. SKUMS. REC. 94. 146). Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

**Funding/Support**
This research was financially supported by Shahrekord University of Medical Sciences, Shahrekord, Iran (grant no. 2640).

**References**
11. Wellington K, Jarvis B. Silymarin: a review of its clinical


