Assessment of hepatoprotective and apoptotic efficacy of
Cynara scolymus leaf extract

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Abstract

Cynara scolymus is an important medicinal plant containing phenolic acids and flavonoids. One of the most active ingredients of cynara is cynarin, which is present in high concentrations, especially in the leaves. Experimental studies demonstrated a significant liver protecting and regenerating effects of cynara but only few of them discussed its anti-apoptotic effects on liver diseases. Forty adult male albino rats were allocated into five groups. Group I: animals served as normal control, Group II: animals received olive oil, Group III: animals received cynara extract, Group IV: animals constituted the hepatotoxic group, which received CCl₄ in olive oil, Group V: CCl₄-prophylactic group administered cynara extract. Blood and liver tissues were collected for biochemical, molecular and histopathological examinations. The CCl₄ group showed significant increases in serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin, albumin, malondialdehyde (MDA), interleukin-6 (IL-6), and decreases in albumin with reduced glutathione (GSH) content and superoxide dismutase (SOD) activity. The co-administration of cynara extract in prophylactic group significantly reduced the elevated levels of biochemical markers mentioned above as well as increased the level of tissue SOD, GSH and serum albumin. Histopathology of liver also confirmed the protective effective of cynara against CCl₄-induced hepatic damage. Gene expression measurements revealed that cynara administration ameliorated the effect of CCl₄ that induced alterations in genes expression of apoptotic related genes Bax, Bel-2 and Caspase-3. The results confirm the strong protective and anti-apoptotic potential of cynara extract against CCl₄-induced hepatic toxicity in rats at both biochemical and molecular levels.

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**Introduction**

Hepatocellular carcinoma (HCC) is the most common primary hepatic malignancy of adults. It is the sixth most common cancer worldwide and the third most common cause of cancer death (Dai et al., 2014). In Egypt, liver cancer forms 11.75% of the malignancies of all digestive organs and 1.68% of the total malignancies. HCC constitutes 70.48% of all liver tumors among Egyptians (Mokhtar et al., 2007). It is the second most common cancer in men and the sixth most common cancer in women (GLOBOCAN database, 2008). HCC represents the main complication of cirrhosis, and shows a growing incidence in Egypt (Gomaa et al., 2014). Pre-existing cirrhosis is an important prerequisite for hepatocarcinogenesis, although some HCCs do arise in the absence of cirrhosis (Gao et al., 2012).

The liver is the largest and most metabolically active and fundamental organ of biotransformation and metabolism (Xu et al., 2011). It is a pivotal organ that removes and inactivates toxic substances and drugs to be excreted in urine. Hepatic toxicity is attributed primarily to the changes in oxidative stress and alteration in acute phase proteins (Flora et al., 2013). This stress induces damage to several biomolecules including lipids, proteins, DNA and, over time, contributes to liver injury. This injury is often differentiated as acute and chronic. Chronic liver disease has continuous formation from steatosis to chronic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (Loguerchio and Federico, 2003 & Vitaglione et al., 2005 & Coballase-Urrutia et al., 2011). The condition of liver is important for our safety and health and its damage or disease is associated with DNA, protein, and lipid damage (Vera-Ramirez et al., 2013).

Hepatotoxicity is defined as injury to the liver that is associated with impaired liver function caused by exposure to a drug or another non-infectious agent (Bahirwani and Reddy, 2014). Chemical toxins including acetaminophen, carbon tetrachloride, galactosamine and thioacetamide are often used as the model substances causing experimental hepatocyte injury in both in vivo and in vitro conditions (Domenicali et al., 2009 & Remien et al., 2014).

Carbon tetrachloride (CCl₄) is a well-known and widely used synthesized chemical to establish animal model to study hepatic injury which is characterized by typical centrilobular necrosis and is similar to the hepatotoxicity in human (Li et al., 2017). It induces toxicity in rat liver, which closely resembles human cirrhosis. CCl₄ induces liver cell injury via the generation of trichloromethyl free radical by the mixed function oxidase system of the endoplasmic reticulum (Jain et al., 2012 & Hu et al., 2014). In addition to trichloromethyl free radical, other toxic products arising from peroxidative degeneration of membrane such as trichloromethylperoxy (OOCCl₃) and chlorine (Cl) free radicals, are responsible for the wide spread hepatocyte damage induced by CCl₄ (Khan et al., 2012). Compared to other body organs, liver is exposed to higher concentration of toxicants, such as CCl₄, and their metabolites because of the dual blood supply from the hepatic artery and the portal vein and high liver content of microsomal cytochrome P₄₅₀ metabolizing enzymes (Canet et al., 2012).

Globe artichoke (Cynara scolymus), belonging to the family of Asteraceae (Compositae), is an herbaceous perennial crop, widely cultivated in the Mediterranean area. Cynara is one of the world’s oldest medicinal plants. It has been known by the ancient Egyptians, while the ancient Greeks and Romans used it as a digestive aid (Magielse et al., 2014). Cynara leaf extract possesses antioxidant properties. It protected cultured rat hepatocytes against hydroperoxide-induced oxidative stress (Ben Salem et al., 2017). Cynara leaf extract also inhibited LDL oxidation (Mileo et al., 2012) and reduced the production of intracellular reactive oxygen species by oxidized LDL in cultured endothelial cells and monocytes (Zapolska-Downar et al., 2002).

Cynara leaves contain phenolic acids, sesquiterpene lactones, flavonoids, phytosterols (taraxasterol), sugars, inulin, enzymes, and essential oils (Simsek and Uysal, 2013). The phenolic acid derivatives include caffeoylquinic acids such as 3-caffeoylquinic acid, cynarin, and caffeic acids. Such properties are consistent with the well-known double role of...
phenolic compounds as antioxidants and as substrates for oxidative browning reactions, primarily in the presence of high iron concentrations (Lattanzio et al., 2009). Chemical activity of polyphenols in terms of their reducing properties, as hydrogen or electron-donating agents, predicts their potential effect as free-radical scavengers (Fratianni et al., 2014). The flavonoid compounds of the leaf are luteolin-7-β-D-glucoside, luteolin-4-β-D-glucoside and luteolin-7-β-rutinoside (ESCOP, 2003 & Lattanzio et al., 2009).

The leaf of the Cynara has been used for centuries as an antimicrobial, anti-inflammatory, choleric, hepatoprotective, cholesterol-lowering, lipid-lowering, and glucose-lowering substance in Turkey, Southern Europe and Mediterranean countries (Jimenez-Escrig et al., 2003).

In view of the above-mentioned beneficial effects of cynara, the aim of this study, therefore, was designed to evaluate the possible protective effect of the cynara leaf extract against CCl₄-induced hepatic injury, as a natural product able to reduce the induced oxidative and apoptotic damage in hepatocytes of male albino rats.

Materials and methods

Cynara preparation

Five % cynarin containing, commercially available cynara leaf extract powder extracted with 75 % ethanol was purchased from Kale Naturel Herbal Products Company, Ltd., Balikesir, Turkey.

Animals

Forty adult male albino rats weighing 180-200 g obtained from the breeding unit of the Medical Research Center (Faculty of Medicine, Ain Shams University, Cairo, Egypt) were used throughout this study. The rats were housed in steel mesh cages on wood-chip bedding and maintained on a commercial pellet diet and tap water for one week before the start of the experiment as an acclimatization period. All animal experiments were performed according to the protocols approved by the local institutional animal ethics committee of Ain Shams University.

Experimental design

The rats were randomly divided into 5 groups of 8 rats each. Group I: animals served as normal control and received distilled water by gavage for 14 days. Group II: animals received olive oil (0.2 ml/kg b.wt.; I.P. twice daily) for 10 days. Group III: animals received cynara extract (1.5 g/kg b.wt.; daily; orally) for 14 days. Group IV: animals constituted the hepatotoxic group, which received CCl₄ in olive oil (0.2 ml/kg b.wt.; I.P twice daily) for 10 days. Group V: prophylactic group administered cynara extract (1.5 g/kg b.wt.; daily; orally) for 14 days and at the last 10 days received CCl₄ in olive oil (0.2 ml/kg b.wt., I.P. twice daily).

Blood and tissue collection

At the end of the experimental period, animals were sacrificed by cervical decapitation. Blood was allowed to clot and then centrifuged to prepare serum that was stored at -20°C. At necropsy, livers were excised immediately, rinsed from blood thoroughly in ice-cold isotonic saline and divided into three parts. The first part of liver (~100 mg) was immediately cut on ice, placed in 10 volumes RNA later (Qiagen, Hilden, Germany) and then stored at 4°C overnight. After the overnight incubation, the RNA later solution was replaced and the liver tissue was preserved at -30°C. The second part of the liver tissue was cut with a surgical blade and fixed in 10% phosphate-buffered formalin for histological examination. The remaining third part of liver was immediately stored in ice-cold sterile saline at -30°C for determination of caspase-3 oxidative stress and antioxidant activities.

Measurements of some biochemical parameters in serum

Serum alanine aminotransferase (ALT; EC 2.6.1.2), aspartate aminotransferase (AST; EC 2.6.1.1) and alkaline phosphatase (ALP; EC 3.1.3.1) activities, as well as total bilirubin level were assayed by the colorimetric methods of Reitman and Frankel (1957), Bessey et al. (1946) and Pearlman and Lee (1974), respectively, using kits purchased from Spectrum (Cairo, Egypt).
Total protein contents in liver homogenates (Lowry et al., 1951) and serum albumin concentration (Doumas et al., 1971) were quantified using kits purchased from Spectrum (Cairo, Egypt).

**Determination of antioxidants and oxidative stress parameters in hepatic tissue**

The liver was weighed and homogenized (10%) in chilled 50 mmol phosphate buffered saline (pH 7.4), centrifuged at 12,000g, at 4°C for 15 min, using universal centrifuge (16R, Germany), then the supernatants were used for the determination of the following parameters: Total (Cu–Zn and Mn) Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed according to the previous described method (Minami and Yoshikawa, 1979), Reduced glutathione (GSH) concentration was measured according to Beutler et al. (1963), Hepatic lipid peroxide (LPO), in terms of malondialdehyde (MDA) was estimated according to the method of Estevez and Cheeseman (1990).

**Determination of serum interleukin-6**

Level of interleukin-6 (IL-6) was determined by using the corresponding commercially available enzyme-linked immunosorbent assay (ELISA) kit following protocols provided by the manufacturers (R&D Systems Kit, USA and DRG, USA).

**Determination of Caspase-3 levels in the liver tissue**

USCN Life Science Inc. Caspase-3 ELISA kit (Cat. No: SEA626Ra) was used to measure caspase-3 concentrations in the liver tissues.

**Histological examination**

Fixed liver samples were embedded in paraffin cubes, sliced into serial 4 µm-thick sections, stained with hematoxylin and eosin (H&E), examined and photographed under a light microscope.

**Quantitative analysis of gene expression by real time PCR**

**Total RNA extraction**

Total RNA was extracted from liver cells using SV Total RNA Isolation System (Promega, Madison, WI, USA) according to manufacturer's instruction.

The RNA concentrations and purity were measured with an ultraviolet spectrophotometer.

**Complementary DNA (cDNA) synthesis**

The cDNA was synthesized from 1µg RNA using SuperScript III First-Strand Synthesis System as described in the manufacturer's protocol (#K1621, Fermentas, Waltham, MA, USA). In brief, 1µg of total RNA was mixed with 50 µM oligo (dT) 20, 50 ng/µL random primers and 10 mM dNTP mix in a total volume of 10 µL. The mixture was incubated at 56°C for 5 min, and then placed on ice for 3 min. The reverse transcriptase master mix containing 2 µL of 10x RT buffer, 4µL of 25 mM MgCl2, 2µL of 0.1 M dithiothreitol (DTT) and 1µL of SuperScript® III RT (200 U/µL) was added to the mixture and was incubated at 25°C for 10 min followed by 50 min at 50°C.

**Real-time quantitative PCR**

Real-time amplification was performed using an Applied Biosystem with software version 3.1 (Stepone™, USA). The reaction containing SYBR Green master mix (Applied Biosystem), gene-specific primer pairs which were shown in Table (1) and were designed with Gene Runner Software (Hasting Software, Inc., Hasting, NY) from RNA sequences from the gene bank. All primer sets had a calculated annealing temperature of 60°C.

Quantitative real-time PCR reactions were performed in a 25 µL reaction volume consisting of 2X SYBR Green PCR master mix (Applied Biosystem), 900 nM of each primer and 2 µL of cDNA. Amplification conditions were: 2 min at 50°C, 10 min at 95°C and 40 cycles of denaturation for 15 sec and annealing/extension at 60°C for 10 min. Data from real-time assays were calculated using the v1.7 sequence detection software from PF Biosystem (Foster City, CA). The relative difference in gene expression of studied gene mRNA between treated groups and the control was determined using the comparative Ct method.

The level of expression of each target gene were normalized to β-actin which was used as the control.
housekeeping gene (endogenous reference gene) and reported as fold change over background levels detected in the diseased groups.

**Statistical analyses**
All data were expressed descriptively as the mean ± standard deviation (SD) of replicate determinations for quantitative parametric data. Statistical analysis was performed using Student t-test to test for differences in means of variables between groups. A probability of $P<0.05$ was considered significant and $P>0.05$ insignificant.

All data were analyzed by Statistical Package for Social Science (SPSS) version 21.0 for Windows (SPSS® Chicago, IL, USA) software program.

**Results**

**Biochemical markers**
No mortality was seen in animals during the study. The dose of cynara did not initiate any side effects for the animals, whereas many side effects were observed in animals treated with CCl$_4$ such as yellowish body hair, loosing of body weight, general weakness (completely loss of activity) and abdominal edema.

### Table 1. Primer sequences of target genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>Forward: 5'-TAGCAAACTGGTGTCAAGG-3'</td>
<td>NM_017059.2</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TCTTGGATCCAGACAAGCAG-3'</td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Forward: 5'-CCAGGCTTCCAACCATTATC-3'</td>
<td>NM_022612.1</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CTCATTGAACCTGCTCCGA-3'</td>
<td></td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Forward: 5'-CAAGTCTGATGGACTGGAA-3'</td>
<td>NM_012922.2</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GTAACATTGCGAGCTGACAT-3'</td>
<td></td>
</tr>
<tr>
<td>$\beta$-actin</td>
<td>Forward: 5'-CTCTGATGCTCCTGAAGTCT-3'</td>
<td>NM_031144.3</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CCATCTCTTTGCTGAAGTCT-3'</td>
<td></td>
</tr>
</tbody>
</table>

**Liver function tests**
The data summarized in Table 2 indicates that, AST, ALT, ALP activities and total bilirubin were significantly increased ($P<0.05$) in rats receiving CCl$_4$ either alone or in combination with cynara extract when compared with the control group. However, the same parameters were significantly lower ($P<0.05$) in prophylactic group when compared to the intoxicated CCl$_4$ group.

In addition, albumin level was significantly decreased in rats received CCl$_4$ when compared with the control group, however, all the other groups showed no significance. For almost all parameters, cynara extract showed normalization of liver function when compared to control group.

**Hepatic oxidative stress**
The hepatic GSH content and SOD activity were significantly decreased ($P<0.05$) in CCl$_4$ and prophylactic groups compared to control group. However, they were significantly increased ($P<0.05$) when cynara extract was co-administered in prophylactic group, compared to CCl$_4$ group. Animals treated with CCl$_4$ had a significant increase ($P<0.05$) in the level of MDA compared to control group. Simultaneous treatment with cynara extract significantly abolished the enhancing effect of CCl$_4$ on hepatic lipid peroxidation that was expressed as a lower level of MDA in hepatocytes ($P<0.05$) (Table 2).

**IL-6 level**
IL-6 level was significantly increased ($P<0.05$) when CCl$_4$ was administered either alone or combined with cynara compared with control group (Table 2).

However, administration of cynara extract alone showed anti-inflammatory effect by decreasing the level of IL-6 significantly ($P<0.05$) when compared to control group. On the other hand, the prophylactic group showed a significant decreased level of IL-6 ($P<0.05$) when compared to the CCl$_4$ group.
Caspase-3 activity
Caspase-3 activity was markedly increased in CCl₄ group \((P<0.05)\), meanwhile co-administration of cynara extract in prophylactic group showed an ameliorating effect by decreasing the level of caspase-3 \((P<0.05)\) in liver tissue when compared to CCl₄ group (Table 2).

Table 2. Biochemical parameters among the studied groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>Control</th>
<th>Olive oil</th>
<th>Cynara</th>
<th>CCl₄</th>
<th>Prophylactic</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td></td>
<td>89.4 ± 7.1</td>
<td>91.3 ± 3.2</td>
<td>108.6 ± 8.6*</td>
<td>316.8 ± 7.7*</td>
<td>93.7 ± 3.7*</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td></td>
<td>39.2 ± 3.4</td>
<td>42.1 ± 2.5</td>
<td>43.2 ± 3.6</td>
<td>187.6 ± 4.6*</td>
<td>59.4 ± 2.4*</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td></td>
<td>97.5 ± 3.1</td>
<td>101.3 ± 4.4</td>
<td>100.6 ± 3.7</td>
<td>274.1 ± 5.6*</td>
<td>112.3 ± 7.7*</td>
</tr>
<tr>
<td>Total Bilirubin (mg/dL)</td>
<td></td>
<td>0.64 ± 0.06</td>
<td>0.59 ± 0.08</td>
<td>0.63 ± 0.03</td>
<td>3.43 ± 0.05*</td>
<td>1.09 ± 0.16*</td>
</tr>
<tr>
<td>Albumin (mg/dL)</td>
<td></td>
<td>4.1 ± 0.39</td>
<td>3.9 ± 0.4</td>
<td>4.2 ± 0.2</td>
<td>2.4 ± 0.6*</td>
<td>3.8 ± 0.9</td>
</tr>
<tr>
<td>MDA (µmol/g tissue)</td>
<td></td>
<td>56.8 ± 4.1</td>
<td>58.1 ± 3.3</td>
<td>55.4 ± 2.9</td>
<td>116.0 ± 3.6*</td>
<td>47.2 ± 4.1*</td>
</tr>
<tr>
<td>GSH (µmol/g tissue)</td>
<td></td>
<td>33.4 ± 1.7</td>
<td>32.6 ± 2.1</td>
<td>33.9 ± 1.8</td>
<td>14.6 ± 0.9*</td>
<td>29.8 ± 1.0*</td>
</tr>
<tr>
<td>SOD (U/mg protein)</td>
<td></td>
<td>2.81 ± 0.5</td>
<td>2.03 ± 0.1</td>
<td>2.70 ± 0.2</td>
<td>1.35 ± 0.4*</td>
<td>2.5 ± 0.3*</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td></td>
<td>38.2 ± 1.3</td>
<td>37.9 ± 1.1</td>
<td>29.4 ± 1.4*</td>
<td>125.1 ± 6.7*</td>
<td>74.6 ± 3.4*</td>
</tr>
<tr>
<td>Caspase 3 (ng/mg protein)</td>
<td></td>
<td>1.34 ± 0.1</td>
<td>1.29 ± 0.09</td>
<td>1.31 ± 0.1</td>
<td>2.9 ± 0.1*</td>
<td>1.62 ± 0.2*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. \(P\) values was statistically measured by independent t-test, \(*P\) value < 0.05 when compared to control group; \#\(P\) value < 0.05 when compared to CCl₄ group.

AST, aspartate transaminase; ALT, alanine transaminase, ALP, alkaline phosphatase; MDA, malondialdehyde; GSH, reduced glutathione; SOD, superoxide dismutase; IL-6, interleukin-6.

Molecular studies
Gene expression of pro-apoptotic (Bax), anti-apoptotic (Bcl-2) and Caspase-3 genes
Bax, Bcl-2 and caspase-3 gene expression were normalized to the housekeeping beta actin gene. As evident from Fig. 1 & 2, real time PCR evaluation of liver cells among the different studied groups showed a significant increase in Bax and caspase-3 mRNA expression in CCl₄ group.

On the contrary, Bcl-2 mRNA expression was relatively decreased in CCl₄ and prophylactic groups, meanwhile cynara extract showed normal pattern of expression. However, the Bax/Bcl-2 ratio appeared to have a tendency to markedly increase in CCl₄ group and to lesser extent in prophylactic group with almost normal level in cynara group compared to that in control group.

Histopathological studies
Histology of the liver sections of control animals showed normal hepatic architecture and liver lobular with well-preserved cytoplasm, prominent nucleus with normal histological structure of the central vein and surrounding hepatocytes in the parenchyma (Fig. 3A). Liver of animals treated with olive oil and cynara extract showed no histopathological alteration and almost normal architecture of hepatic lobules as recorded in Fig. 3B & 3C, respectively. The liver sections of CCl₄-treated animals showed accumulation of adipoblasts with fibrosis while the underlying hepatocytes had degenerative change in Fig. 3D. Dilatation was observed in the central vein also associated with fatty change in the surrounding few hepatocytes in Fig. 3E. Finally, mild thickening was detected in the capsule associated with degeneration in the underlying hepatocytes in group of cynara intoxicated with CCl₄ in Fig. 3F.
Discussion

In absence of a reliable and effective agent for prevention and treatment of liver diseases, many researchers are focusing on introducing hepatoprotective compounds from natural products (Kumar et al., 2011). Many epidemiological studies suggest that diet rich in fruits and vegetables has healthy properties (Mileo et al., 2016). The phenolic and flavonoid contents of these natural products have many advantages either through scavenging the free radicals (Kalaiselvan et al., 2016) or exhibiting some apoptotic events (Supic et al., 2013).

Artichoke (Cynara scolymus) is a healthy food and folk medicine with antioxidative and anti-inflammatory properties. Although the most important active ingredient of cynara, cynarin, is present in the whole plant, the highest concentrations are found in the leaves. For this reason, most of the natural medicines obtained from this plant are prepared from leaves. Like silymarin, cynarin has demonstrated significant liver protecting and regenerating effects (Moglia et al., 2008).

Liver function tests

One of the ways for estimating of the extent of hepatic damage is through the determination of the serum level of cytoplasmic enzymes that leak from damaged liver cells into the blood stream. The results of the present work indicated that the administration of CCl₄ caused severe liver injury which was evidenced by the increase in the activities of serum hepatic enzymes AST, ALT, ALP and total bilirubin accompanied with decrease in the albumin content. This may be due to the fact that hepatic cells posses a variety of metabolic activities and in case of liver injury the transport function of hepatocytes is disturbed, resulting in the leakage of these metabolites (Biswas et al., 2010). Also, these results are in agreement to previous studies, showing that increased bilirubin levels indicated diffused harm to the liver (El-Kott and Owayss, 2008).

At the same time, the current results showed a pronounced decrease in the activities of serum ALT, AST, ALP and total bilirubin after oral treatment with prophylactic dose of cynara extract when compared to the CCl₄ group. This effect of cynara is in agreement with previous study (Heidarian and Rafieian-Kopaei, 2013) which stated that cynara significantly improved the liver status and showed more profound therapeutic effects. This protective effect may be due to the antioxidant effect of cynara which was previously confirmed (Desai et al., 2012).
Another study (Header et al., 2017) reported that the treatment with cynara extract significantly prevented the release of transaminases and significantly enhanced protein content towards control, suggesting its hepatoprotective potential. The pharmacological activities of cynara extract as hepatoprotective, antioxidative, anticarcinogenic, hypocholesterolemic and antibacterial effects, which may be attributed to the presence of phenolic and flavonoid compounds can be useful in the treatment of liver damage (Gouveia and Castilho, 2012).

**Hepatic oxidative stress**

Lipid peroxidation produces a progressive loss of cell membrane integrity, impairment in membrane transport function and disruption of cellular ion homeostasis (Bano and Bhatt, 2007). The current study revealed that the administration of CCl₄ resulted in a significant increase in the liver MDA content which is the most important oxidative stress parameters in the liver. These results are in agreement with the previous study which confirmed the liver damage of CCl₄ through the higher level of MDA which directly stimulated the activation of hepatic stellate cells and accelerated liver fibrosis (Guo et al., 2017). On the contrary, it has been suggested that therapeutic activities of cynara extract depend mainly on the presence of phenolics and flavonoids. These compounds are known for their strong scavenging effect on free radicals and may also be able to suppress the formation of free radicals by binding to heavy metal ions which are known to catalyze many processes leading to the generation of free radicals.

In the present study, cynara extract attenuated the CCl₄-induced MDA formation possibly due to its intrinsic antioxidant properties, thus cynara extract may prevent peroxidative changes in liver tissue. These results are in agreement with previous study (Colak et al., 2016) that also documented the effect of cynara through the renewal mechanism of the liver by the re-stabilization of the cell membranes and repair mechanisms in the hepatic tissue as a result of recovery against the oxidative stress.

Furthermore, the accumulation of free radicals may cause decrease in antioxidant capacity such as a decline in SOD and GSH levels and hence diminished capacity of scavenging free radicals (Wang et al., 2003). In the present study, the decrease of the GSH and SOD content of the liver tissues after the application of CCl₄ have also been described previously (El-Sayed et al. 2015). On the other hand, the present study showed significant improvement in GSH content.

![Fig. 2. Quantitative RT-PCR analysis of Bax/Bcl-2 ratio mRNA expressions in liver of the studied groups. Data are expressed as mean ± SD. *P values was statistically measured by independent t-test, *P value < 0.05 when compared to control group; # P value < 0.05 when compared to CCl₄ group.](image)
nd SOD activity in rats protected by cynara extract. This improvement may be due to its rich content of essential elements such as K, Ca, Na, Mg, I, Mn, Zn, Cu and Cr and some vitamins such as A, B1, B2, B3 and C, that might be responsible for reactivating antioxidant enzymes by providing optimum trace elements and so enhancing the SOD and GSH levels (Monti et al., 2008).

![Histological investigations of hematoxylin and eosin-stained liver tissue sections. A; Control group (10X), B; Olive oil group (10X), C; Cynara leaf extract group (10X), D, E; CCl₄-induced group (20X), F; Prophylactic group (10X).](image)

**IL-6 level**
The production of some pro-inflammatory cytokines by hepatic Kupffer cells is increased in acute inflammatory responses. These cytokines can trigger hepatic stellate cells and promote their production; therefore, enhancing extracellular matrix (ECM) deposition and exacerbating the fibrogenesis process (Friedman, 2008). In the present study, CCl₄ administration caused an increase in the levels of IL-6 compared to control group. These results are in agreement with previous studies that reported the capability of CCl₄ to induce some inflammatory responses by elevation of some cytokines such as IL-6 (Hassan et al., 2012) and activation of caspase cascade of apoptosis as indicated by the increase in activity of active caspase-3 (Yao et al., 2017).

Meantime, cynara extract exhibited an ameliorating effect which may be due to the unique active constituents of cynara that have been shown to exert a variety of medical properties including anti-inflammatory activity (Mocelin et al., 2016).

**Caspase-3 activity**
Cell apoptosis can be induced by the activation of death receptors, mitochondrial dysfunction, DNA damage, excessive autophagy, and endoplasmic reticulum stress.

In liver cells, mitochondrial dysfunction can amplify the apoptotic signal and integrate various pathways to promote apoptotic response (Fernandez et al., 2015). Caspase-3 is a 32 kDa caspase protein that interacts with caspase-8 and caspase-9 and has a central role in the execution-phase of cell apoptosis. Data of the current study demonstrated that CCl₄ induced high levels of caspase-3 in the rat liver.
This result suggested the capability of CCl₄ to induce the apoptosis as previously proved by El-Halawany et al. (2014). Meanwhile, the pretreatment of cynara leaf extract inhibited caspase-3 activity in prophylactic group which was elevated after the administration of CCl₄ and protect against hepatic damage via an anti-apoptotic function. These results are in agreement with the previous study of Colak et al. (2016).

**Molecular studies**

Furthermore, the dramatic effects of CCl₄ and the valuable effect of cynara extract on the activity of liver enzymes, antioxidant, inflammatory and apoptotic markers are supported by the molecular studies on gene expression of anti-apoptotic (Bcl-2), pro-apoptotic (Bax) and caspase-3 genes. Bax and Bcl-2 have been reported to play a major role in determining cellular fate under injured conditions. Increased expression of Bax can induce apoptosis, while upregulation of Bcl-2 protein protects cells (Tallman et al., 2005).

In the current study, the expression levels of Bcl-2, Bax, and caspase-3 were investigated by quantitative real time PCR. As expected, CCl₄ treatment increased the Bax expression and downregulated the Bcl-2 expression (Hamid et al., 2017), which could be reversed by cynara extract effect. Meanwhile, the expression of caspase-3 was increased by the treatment with CCl₄ and marvelous decreased by cynara.

These results support that cynara protects the liver against hepatocyte apoptosis during CCl₄-induced liver injury as previously reported by Mileo et al. (2012). Also, as stated previously, cynara polyphenols can act over different stages of apoptosis by modifying Bcl-2 and Bax expression levels (Jahanafroz et al., 2016). The ultimate vulnerability of cells to diverse apoptotic stimuli is determined by the relative ratio of various pro-apoptotic and anti-apoptotic members.

The balance between Bax and Bcl-2 expression levels help to determine the susceptibility of a cell to apoptotic stimuli.

The present work confirmed that co-administration of cynara extract with CCl₄ significantly decreased the expression of Bax/Bcl-2 mRNA ratio compared with CCl₄ group. This result agreed with the earlier work of Kakkar and Singh (2007) that showed that cells’ survival is frequently regulated by pro-apoptotic and anti-apoptotic family proteins. A similar result was also reported by Ding et al. (2010).

**Histopathological studies**

In terms of histopathological changes, several previous experimental studies have reported that administration of CCl₄ led to a variety of structural changes in the liver tissue such as inflammation, apoptosis, fibrosis, fatty degeneration, mononuclear cell infiltration, steatosis, hemorrhage, formation of regenerative nodules in liver tissue, sinusoid dilation, vacuolisation and centrilobular necrosis (Kim et al., 2014). The present study support these documented findings in CCl₄ group. Following the CCl₄-induced hepatic injury, the administration of cynara leaf extract led to reduction of these histopathological changes where mild thickening were detected in prophylactic group, meanwhile there was no histopathological alterations were observed in cynara group. These findings are in agreement with earlier study that confirmed the ameliorating effect of cynara in rat liver which showed a mild degree of lymphocyte infiltration compared to the control (Heidarian and Rafieian-Kopaei, 2013).

**Conclusion**

In conclusion, according to all the achieved biochemical, molecular and histopathological findings in the present study, cynara extract can prevent or alleviate the liver injury induced by CCl₄ in rats towards the normal state with the reversal of altered antioxidant enzymes, apoptotic status and peroxidation damage in tissues.

These confirmed its hepatoprotective effect and potential role in defense against free radicals. The protective effects may be, at least in part, related to the antioxidant and anti-apoptotic properties of the extract that may be due to its content of the phenolic acids and flavonoids.
These results suggested the pharmaceutical phytomedicine role of cynara leaf extract in the treatment of liver diseases.

References


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