Hepatoprotective Effect of Artichoke Extract against Pre-cancerous Lesion of Experimentally Induced Hepatocellular Carcinoma in Rats

Ramadan, A.1, Nehal A. Afifi1, Nemat Z. Yassin2, Rehab F. Abdel-Rahman2, Azza H. M. Hassan3 and Hany M. Fayed2

1Pharmacology Department, Faculty of Veterinary Medicine, Cairo University, Egypt
2Pharmacology Department, Medical Division, National Research Center, Egypt
3Pathology Department, Faculty of Veterinary Medicine, Cairo University, Egypt
azzahassan99@gmail.com

Abstract: Hepatocellular carcinoma (HCC) is a primary malignancy of the liver. It is now the third leading cause of cancer deaths worldwide. The incidence of HCC is highest in Asia and Africa, where the endemic high prevalence of hepatitis B and hepatitis C strongly predisposes to the development of chronic liver disease and subsequent development of HCC. The present study was conducted to investigate the protective effect of Cynara scolymus leaves extract against N-nitrosodiethylamine (NDEA) at a dose of 100 mg/kg b.wt and carbon tetrachloride (CCl4) at a dose of 3ml/kg b.wt -induced hepatocarcinogenesis in male Wistar albino rats. Main methods: rats were pretreated with Cynara scolymus extract, silymarin or both for six weeks prior to the injection of NDEA. Then rats administered with a single intraperitoneal injection of NDEA followed by subcutaneous injections of CCl4 once a week for 6 weeks and the pretreatment was continued for another six weeks. At the end of the experiment, blood samples were taken for biochemical analysis and liver tissues were histopathologically examined. Results of the current study showed significant increase in serum levels of AST, ALT, ALP, total and direct bilirubin and alpha fetoprotein (AFP) as well as significant decrease in albumin was observed in NDEA-intoxicated group, compared to normal control group. Pretreatment with Cynara scolymus extract, silymarin and their combination limited the increase in serum levels of AST, ALT, ALP, total and direct bilirubin and alpha fetoprotein (AFP) and produced significant increase in albumin, compared to NDEA- intoxicated group. Histopathological examination of liver of NDEA and CCl4 treated group revealed obvious cellular damage and hepatocellular carcinoma with frequent mitotic activity and nuclear pleomorphism, meanwhile other groups pretreated with either silymarin, Cynara scolymus extract or a combination of silymarin and Cynara scolymus extract revealed improvement in the liver architecture. The current results indicated that Cynara scolymus extract possess hepatoprotective effect.

1. Introduction

Among primary liver cancers, hepatocellular carcinoma (HCC) represents the major histological subtype, accounting for 70% to 85% of the total liver cancer burden worldwide (Wang et al., 2013). Globally, it is the fifth most common cancer and the second leading cause of cancer-related death (Zhang et al., 2013). In Egypt, hepatocellular carcinoma contributes to 14.8% of all cancer mortality with a higher incidence in males (13.6%) than in females (11.5%). It is the second most frequent cancer type in Egyptian males after bladder cancer and the eighth most frequent in Egyptian females (Aleem et al., 2012). Chronic inflammations of the liver and subsequent cirrhosis are highly correlated with hepatitis B and hepatitis C viral infections, alcohol abuse and metabolic liver diseases (diabetes and non alcoholic fatty liver disease). Additionally, obesity, environmental pollutants, consumption of food contaminated with the fungal toxins as aflatoxins that is produced by Aspergillus flavus in food grains and nitrosamine consumption are the strongest risk factors for HCC development (Farazi et al., 2006).

Food plants, including fruits, vegetables, and spices are the primary sources of naturally occurring nutrients essential for human health. Due to their health benefits, vegetables and fruits have become popular among consumers and the number of medicinal plants being used in healthcare has increased worldwide. Phytochemicals with antioxidative, anti-inflammatory, antibacterial and antimutagenic properties are extremely attractive potential agents for preventing diseases in humans (Li et al., 2012).

Cynara scolymus L. is an ancient herbaceous plant, originating from the Mediterranean area. Today, it is widely cultivated all over the world. Its leaf is widely used for therapeutic purposes (Metwally et al.,
Cynareae tribe present in Egypt are known for their efficacy in relieving some liver disorders (El-Sohafy et al., 2013). Artichoke (Cynara scolymus) leaf extracts have traditionally been used to treat dyspeptic symptoms, increases bile flow and to exert hepatoprotective, lipid-lowering, antioxidant and antispasmodic effects (Holtmann et al., 2003).

The aim of the present study was to investigate the hepatoprotective effect of Cynara scolymus extract against N-nitrosodiethylamine (NDEA) and carbon tetrachloride (CCl4) induced hepatocarcinogenesis in rats, comparing with silymarin as a standard drug.

2. Material and Methods

Rats and Diet:
Sixty; adult male Wistar albino rats, 200-250 g were obtained from the animal house colony, National Research Centre, Giza, Egypt.

All animals were housed in metal cages in a well-ventilated environment at (22 ± 3°C, 55 ± 5% humidity and 12h dark & light cycles); received standard rat food pellets and water was provided ad libitum throughout the experimental period.

All experiments were carried out according to the ethical guidelines for care and use of experimental animals approved by the Ethical Committee of the National Research Centre.

Chemicals:
N-Nitrosodiethylamine (NDEA) was purchased from Sigma Chemical Company, USA. Carbon tetrachloride (CCl4) was obtained from El-Gomhorya Company, Cairo, Egypt. Biochemical kits for serum analysis were purchased from the Gamma Trade Company, Cairo, Egypt. Biochemical kits for serum analysis were purchased from the Gamma Trade Company for Pharmaceutical and Chemicals, Dokki, Egypt.

Plants:
Cynara scolymus dry extract and silymarin were purchased from MEPACO, Egypt as a fine powder and stored in an airtight container in a refrigerator below 10 °C.

Experimental Design:
Adult male Wistar albino rats weighing 200–250 g (10–12 weeks old) were divided into six groups. Group I (Normal control), rats were IP injected with Dimethyl sulfoxide (DMSO) at a dose of 1 ml/kg and injected SC with liquid paraffin (3 ml/kg). The other five groups were given a single IP injection of N-nitrosodiethylamine (NDEA; 100 mg/kg b.wt.) followed by weekly SC injections of CCl4 (3ml/kg b.wt.) for six weeks as reported by (Sundaresan et al., 2003) and then divided as follow: Group II Kept as hepatotoxic group. Group III (standard group) was given silymarin orally at a dose of 50 mg/Kg b.wt. per day for six weeks before induction of hepatocarcinogenesis and continued for another six weeks (Shaarawy et al., 2009). Groups IV and V were orally administered Cynara scolymus leaves extract at doses of (750 and 1500 mg/kg b.wt) per day for six weeks before induction of hepatocarcinogenesis and continued for another six weeks (Mehmetcik et al., 2008). Groups VI, rats were received Cynara scolymus extract at a dose of 750 mg /kg b.wt in combination with silymarin (50mg/kg b.wt) per day orally for six weeks before induction of hepatocarcinogenesis and continued for another six weeks. At the end of the experimental period (12 weeks), blood samples were collected from the retro-orbital venous plexus of rats under light ether anesthesia and collected in clean test tubes, allowed to clot, then centrifuged for 10 minutes at 3000 r.p.m. Serum was separated and stored into Eppendorff tubes at – 20 °C to be used for determination of liver function parameters.

Determination of liver function tests:
Serum activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined colorimetrically according to the method of (Reitman et al., 1957). Serum alkaline phosphatase (ALP) was calorimetrically determined according to the method described by (Babson et al., 1966). Serum direct and total bilirubin determined colorimetrically as described by (Doumas et al., 1973). Serum concentration of albumin was determined as described by (Bartholomew et al., 1966).

Determination of serum alpha fetoprotein (AFP) (as a tumor marker):
Serum levels of alpha fetoprotein (AFP) were quantified as performed by (Gibbs et al., 1987) using an enzyme-linked immunosorbent assay (ELISA) kit.

Histopathological examination:
For histopathological study, few-millimeters mid-sections of the left lobes of the liver excised from each group were processed for light microscopy. The processing involved fixing the tissue specimens in a 10% neutral buffered formalin solution, preparing the blocks in paraffin, cutting sections 5-6 μm in thickness, and staining the sections with haematoxylin-eosin stain. The sections were examined by an expert pathologist who was not aware of sample assignment to experimental groups (Carleton et al., 1967).

Statistical analysis:
In the present study, all results were expressed as mean ± standard error of the mean. Data analysis was achieved by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using software program GraphPad Prism (version 5.00). Difference was considered significant when P<0.05.
3. Results:

The results in Table (1) showed that administration of a single toxic dose of N-nitrosodiethylamine (NDEA; 100 mg/kg b.wt) followed by a weekly S.C. injections of CCl4 (3ml/kg) for 6 weeks induced a significant increase in serum AST, ALT and ALP levels when compared to the normal control group.

Pretreatment of rats with *Cynara scolymus* extract at doses of 750 and 1500 mg/kg b.wt, silymarin and their combination limited the elevation of serum AST, ALT and ALP levels when compared to NDEA-intoxicated group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg b.wt)</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>ALP (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>-</td>
<td>97.60±1.36</td>
<td>38.53±1.08</td>
<td>124.20±2.88</td>
</tr>
<tr>
<td>NDEA–intoxicated rats</td>
<td>100</td>
<td>391.70±2.93</td>
<td>171.30±4.31</td>
<td>296.10±0.85</td>
</tr>
<tr>
<td>NDEA–intoxicated rats + Artichoke extract</td>
<td>750</td>
<td>230.20±9.44</td>
<td>100.80±1.86</td>
<td>168.50±4.16</td>
</tr>
<tr>
<td>NDEA–intoxicated rats + Artichoke extract</td>
<td>1500</td>
<td>113.20±2.57</td>
<td>50.73±1.07</td>
<td>140.50±3.07</td>
</tr>
<tr>
<td>NDEA–intoxicated rats treated with Artichoke extract and Silymarin</td>
<td>750 50</td>
<td>111.60±3.42</td>
<td>47.63±1.81</td>
<td>134.30±3.24</td>
</tr>
<tr>
<td>NDEA–intoxicated rats + Silymarin</td>
<td>50</td>
<td>113.20±2.48</td>
<td>48.37±1.46</td>
<td>135.70±1.72</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± S.E. for each group.

P <0.05: Statistically significant from control group (one way ANOVA followed by "Tukey's Multiple Comparison Test").

P<0.05: Statistically significant from NDEA–intoxicated group (one way ANOVA followed by "Tukey's Multiple Comparison Test").

The results in Table (2) showed that serum level of direct and total bilirubin significantly elevated in NDEA–intoxicated group, as compared to the normal control group.

Rats pretreated with *Cynara scolymus* extract (750 and 1500 mg/kg b.wt), silymarin and their combination prevented the elevation of serum direct and total bilirubin as compared with NDEA–intoxicated group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg b.wt)</th>
<th>Direct Bilirubin (mg/dl)</th>
<th>Total Bilirubin (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>-</td>
<td>0.12±0.007</td>
<td>0.32±0.01</td>
</tr>
<tr>
<td>NDEA–intoxicated rats</td>
<td>100</td>
<td>2.52±0.09</td>
<td>3.81±0.08</td>
</tr>
<tr>
<td>NDEA–intoxicated rats + Artichoke extract</td>
<td>750</td>
<td>1.46±0.04</td>
<td>1.80±0.04</td>
</tr>
<tr>
<td>NDEA–intoxicated rats + Artichoke extract</td>
<td>1500</td>
<td>0.36±0.02</td>
<td>0.42±0.02</td>
</tr>
<tr>
<td>NDEA–intoxicated rats treated with Artichoke extract and Silymarin</td>
<td>750 50</td>
<td>0.23±0.01</td>
<td>0.27±0.01</td>
</tr>
<tr>
<td>NDEA–intoxicated rats + Silymarin</td>
<td>50</td>
<td>0.30±0.01</td>
<td>0.39±0.01</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± S.E. for each group.

P <0.05: Statistically significant from control group (one way ANOVA followed by "Tukey's Multiple Comparison Test").

P<0.05: Statistically significant from NDEA–intoxicated group (one way ANOVA followed by "Tukey's Multiple Comparison Test").

The results in Table (3) showed that rats injected with NDEA and CCL4 caused a significant reduction in serum albumin level as compared with the normal control rats.

Groups pretreated with *Cynara scolymus* extract (750 and 1500 mg/kg b.wt), silymarin and their combination maintained synthetic capacity of hepatocytes which exhibited by significant elevation of serum albumin as compared with NDEA–intoxicated group.
Table 3: Effect of oral administration of *Cynara Scolymus* extract on serum albumin level.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg b.wt)</th>
<th>Albumin (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>-</td>
<td>5.62±0.18</td>
</tr>
<tr>
<td>NDEA–intoxicated rats</td>
<td>100</td>
<td>2.73±0.07</td>
</tr>
<tr>
<td>NDEA–intoxicated rats + Artichoke extract</td>
<td>750</td>
<td>4.50±0.14</td>
</tr>
<tr>
<td>NDEA–intoxicated rats + Artichoke extract</td>
<td>1500</td>
<td>5.60±0.16</td>
</tr>
<tr>
<td>NDEA–intoxicated rats treated with Artichoke extract and Silymarin</td>
<td>750</td>
<td>5.77±0.26</td>
</tr>
<tr>
<td>NDEA–intoxicated rats + Silymarin</td>
<td>50</td>
<td>5.58±0.22</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± S.E. of (n=5) for each group.

P<0.05: Statistically significant from control group (one way ANOVA followed by "Tukey's Multiple Comparison Test").

P<0.05: Statistically significant from NDEA –intoxicated group (one way ANOVA followed by "Tukey's Multiple Comparison Test").

The results in Table (4) showed a significant increase in serum α-fetoprotein (AFP) level in NDEA- intoxicated group, as compared to normal control group.

Table 4: Effect of oral administration of *Cynara Scolymus* leaves extract on serum AFP level.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg b.wt)</th>
<th>α-fetoprotein (AFP) (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>-</td>
<td>2.98±0.06</td>
</tr>
<tr>
<td>NDEA–intoxicated rats</td>
<td>100</td>
<td>8.06±0.10</td>
</tr>
<tr>
<td>NDEA–intoxicated rats + Artichoke extract</td>
<td>750</td>
<td>4.02±0.04</td>
</tr>
<tr>
<td>NDEA–intoxicated rats + Artichoke extract</td>
<td>1500</td>
<td>3.62±0.08</td>
</tr>
<tr>
<td>NDEA–intoxicated rats treated with Artichoke extract and Silymarin</td>
<td>750</td>
<td>3.55±0.04</td>
</tr>
<tr>
<td>NDEA–intoxicated rats + Silymarin</td>
<td>50</td>
<td>3.60±0.08</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± S.E. for each group.

P<0.05: Statistically significant from control group (one way ANOVA followed by "Tukey's Multiple Comparison Test").

P<0.05: Statistically significant from NDEA –intoxicated group (one way ANOVA followed by "Tukey's Multiple Comparison Test").

Histopathological examination of liver sections of control rats showed normal hepatic architecture. On the other hand, liver of NDEA and Ccl4 treated group revealed marked disarrangement of hepatic lobules with characteristic hepatocellular carcinoma (HCC) formation in which the tumor cells arranged in a compact pattern (Fig.1). The tumor cells showed large hyperchromatic nuclei with prominent nucleoli, nuclear pleomorphism and frequent mitotic activity as well as presence of large amount of cytoplasmic fat and/or glycogen (Fig.3&3) that result in a clear cell appearance. Portal area revealed intense oval cell hyperplasia that arranged themselves in acinar like structure associated with lymphocytic cell infiltration (Fig.4). While Liver sections of rats pretreated with silymarin revealed less disarrangement of hepatic architecture with minimal mitosis and nuclear prominence, as well as presence of intracytoplasmic fat and/or glycogen globules especially at the periportal area (Fig.5). Liver sections of rats pretreated with *Cynara Scolymus* extract 750 and 1500 mg/kg b.wt showed nearly the same histopathological alterations represented by preserved hepatic architecture, minimal nuclear changes, vacuolation of hepatocellular cytoplasm and scanty mitosis (Fig.6&7). Liver sections of rats pretreated with a combination of *Cynara Scolymus* and silymarin restored many of normal hepatic architecture with less disarrangement and degeneration of hepatocytes, in addition to apoptosis and single cell necrosis (Fig.8) compared with NDEA treated group.
Fig. 1: Liver of NDEA and CCl₄ treated rat showing compact hepatocellular carcinoma (HCC) (H&E x200).
Fig. 2: Liver of NDEA treated rat showing large hyperchromatic nuclei with prominent nucleoli, nuclear pleomorphism and frequent mitotic activity (arrow) (H&E x400).
Fig. 3: Liver of NDEA treated rat showing cytoplasmic fat and/or glycogen as well as mitotic activity (H&E x400).
Fig. 4: Liver of NDEA treated rat showing intense oval cell hyperplasia arranged in acinar like structure (arrow) associated with lymphocytic cell infiltration (H&E x400).
Fig. 5: Liver of rat pretreated with silymarin showing intracytoplasmic fat and/or glycogen globules (H&E x200).
Fig. 6: Liver of rat pretreated with *Cynara scolymus* at a dose of 750 mg/kg b.wt showing preserved hepatic architecture, minimal nuclear changes and vacuolation of hepatocellular cytoplasm (H&E x200).
Fig. 7: Liver of rat pretreated with *Cynara scolymus* at a dose of 1500 mg/kg b.wt vacuolation of hepatocytes (H&E x400).
Fig. 8: Liver of rat pretreated with *Cynara scolymus* and silymarin showing vacuolar degeneration of hepatocytes, apoptosis (arrow) and single cell necrosis (H&E x400).

4. Discussion:
In the present study, NDEA and CCl₄ administration to rats led to a marked increase in the levels of serum AST, ALT and ALP compared to the normal group, which indicating that NDEA could induce a liver damage in the rats. These results are in
agreement with Bansal et al. (2005) who attributed the elevation of serum transaminases and alkaline phosphatase to the injured structural integrity of the liver as these enzymes released from the cytoplasm into the blood circulation after rupture of the plasma membrane and cellular damage. These results are also in agreement with Mittal et al. (2006) who found that activities of AST, ALT and ALP were increased significantly following nitroso compounds treatment in rats due to substantial liver damage.

In the present investigation, there was a marked increase in serum levels of direct and total bilirubin as compared to the normal group. These findings are in agreement with the data presented by Rezaie et al. (2013) who revealed a highly significant increase in serum level of both total and direct bilirubin in NDEA treated rats. These results may be due to inhibition of the conjugation reaction and release of unconjugated bilirubin from damaged hepatocytes Rajkapaor et al. (2006). Concerning the level of serum albumin, the current study showed a highly significant decrease in their level after administration of NDEA compound. These results supported with Cross et al. (1987) who attributed the decreased in albumin level to the increased rate of catabolism rather than impairment of synthesis due to the highly toxic effect of carcinogen, which leads to increase in formation of reactive oxygen species (ROS) that are capable of damaging biological molecules such as proteins. Vandenberghe (1996) explained that hypoalbuminemia may be due to kidney function disorder caused by the toxic effect of NDEA which leads to increase in albumin loss.

In the current study, daily administration of Cynara scolymus extract (0.75g/kg, 1.5g/kg), silymarin and their combination significantly limited the elevation of serum AST, ALT and ALP levels compared to NDEA intoxicated group. These findings are in agreement with Speroni et al. (2003) who revealed that these effects due to the active ingredients of Cynara scolymus which are known as caffeoylquinic acid derivatives (cynarin and chlorogenic acid) via their strong hepatoprotective effect and antioxidant capacity.

In this study, daily administration of Cynara scolymus extract (0.75g/kg, 1.5g/kg), silymarin and their combination significantly limited the elevation of serum total and direct bilirubin levels. These results are in agreement with Ghanem et al. (2009) who attributed this reduction in serum bilirubin to cynarin which stimulates the clearance of bile from the liver, preventing congestion in the liver and diminishing the liver damage.

Administration of Cynara scolymus extract (0.75g/kg, 1.5g/kg), silymarin and their combination significantly increased the level of serum albumin compared with NDEA intoxicated rats. These results are in agreement with El Saeed et al. (2012) who attributed this elevation of serum albumin to the anabolic effect of flavonoids. Also Jimenez-Escrig et al. (2003) explained that the hepatoprotective activity of artichoke may play a role by increasing biosynthesis of proteins (including albumin) by the liver.

Serum AFP level was significantly increased in NDEA-intoxicated group as compared to the normal group. The obtained results are in agreement with those obtained by Song et al. (2013); they reported that NDEA administration led to increased level of alpha-fetoprotein. Motaleb et al. (2008) attributed the reinitiation of AFP synthesis by neoplastic hepatocytes to increased transcription of AFP gene. The present study showed that daily administration of Cynara scolymus extract at doses of (0.75 and 1.5g/kg b.wt), silymarin and their combination significantly limited the elevation of serum AFP level as compared to NDEA-intoxicated group, suggesting that Cynara scolymus extract might delay the NDEA-induced HCC in rats.

Histopathology of the liver tissue of NDEA and Ccl4 treated group revealed hepatocellular carcinoma with cellular and nuclear pleomorphism and frequent mitosis. These alterations could be attributed to the toxic effect of NDEA through generation of reactive oxygen species (ROS) with production of malondialdehyde (MDA) and 4-hydroxynonenal (Metwally etal,2011) which attack cellular targets including DNA, thereby inducing mutagenicity and carcinogenicity (Jeyabal etal,2005). Liver sections of rats pretreated with silymarin revealed improvement in the hepatocytes that exhibited less disarrangement and few mitosis. This improvement could be attributed to the antioxidant activity of silymarin which reduce intracellular ROS level of hepatocytes ,preventing oxidative stress-induced cellular damage (El Mesallamy etal,2011). Liver sections of rats pretreated with Cynara scolymus revealed marked improvement in the hepatocytes compared with NDEA treated group. This improvement may be attributed to the antioxidant capacity of Cynara scolymus and suppression of tumor promotion (Yasukawa et al,2012).

**Conclusion:**

From this study it can be concluded that Cynara scolymus extract possess hepatoprotective activity and the protective effect of cynara scolymus extract at a dose of (1.5g/kg b.wt) was more prominent than that of (0.75g/kg b.wt). However, the combination of Cynara scolymus extract at a dose of (0.75g/kg b.wt) and silymarin was the strongest among other groups. Therefore it is recommended to use Cynara scolymus as food supplements in patients with chronic liver diseases.
Corresponding Author
Dr. Azza H. M. Hassan
Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Egypt.
azzahassan99@gmail.com

References