Clinicopathological and histopathological studies on the hepatoprotective effect of artichoke in albino rats


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Abstract:
The present study was conducted to evaluate the protective effect of artichoke on Diethylnitrosamine (DEN) induced oxidative stress and hepatic fibrosis in male albino rats. Forty male albino rats were divided into 4 equal groups. Group (I) was kept as control. Group (II) received artichoke 300mg/kg b. wt. orally via stomach gavage daily for 14 weeks. Group (III) injected intrapretoneally with DEN 50mg/kg b.wt. once weekly for 12 weeks. Group (IV) received artichoke via stomach gavage 300 mg/kg b.wt, two weeks prior to DEN injection and at the beginning of the 3rd week the rats were intrapretoneally injected with DEN 50mg/kg b.wt once weekly. All rats were killed at the end of 14th week. DEN administration resulted in significant elevation of liver enzymes as well as hepatic malondialdehyde (MDA). Glutathione (GSH) level, superoxide dismutase (SOD) and catalase (CAT) activities were also increased. In rats pretreated with artichoke extract (group IV), significant decreases in liver enzymes activities and MDA were observed following DEN treatment as compared to DEN-treated rats, but GSH level, SOD & CAT activities were increased. The present clinicopathological and histopathological findings indicated that artichoke significantly reduced the liver toxicity. So it could be used as hepatoprotective agents against hepatotoxicity.

Introduction:
Liver fibrosis is a common response to chronic liver injury, manifested as hepatocyte necrosis, regeneration and collagen deposition, which is reversible at best and, at worst, may result in cirrhosis that is irreversible (Friedman, 2003). Artichoke (Cynara scolymus L.) is a plant that is widely grown in Mediterranean countries and is rich in natural antioxidants. It contains caffeoylquinic acid derivatives (cynarin and chlorogenic acid) and flavonoids (luteolin, apigenin) (Joy and Haber, 2007). The artichoke
leaf extract has been used for hepatoprotection (Speroni et al., 2003). In vivo studies have shown that artichoke extract is very effective as an antioxidant and its health-protective potential has been attributed to its antioxidant power (Jimenez-Escrig et al., 2003). It has been found to decrease the production of reactive oxygen species (Zapolska-Downar et al., 2002), lipid peroxidation (Speroni et al., 2003), protein oxidation (Jimenez-Escrig et al., 2003). Although it has been reported that artichoke may have protective effects against liver injury (Speroni et al., 2003), the experimental studies are insufficient.

Diethylnitrosamine (DEN) is an N-nitroso alkyl compound described as an effective hepatotoxic agent (Jose et al., 1998). DEN is found in a wide variety of foods such as cheese, soybeans, smoked, salted and dried fish, cured meat and alcoholic beverages (Liao et al., 2001). Metabolism of certain therapeutic drugs is also reported to produce DEN (Akintonwa 1985). DEN has been suggested to cause oxidative stress and cellular injury due to involvement of free radicals (Noguchi et al., 2000). Oxidative stress is considered as a critical mechanism contributing to DEN-induced hepatotoxicity, so the use of antioxidant agents reduced liver damage (Vitaglione et al., 2004). The present study aimed to evaluate the hepatoprotective effect of artichoke extract against diethylnitrosamine-induced hepatotoxicity.

Materials and methods:
Animals: forty male albino rats of body weight 100-150 gm were used in this study. Rats were obtained from National Research Center, Cairo. The animals were kept in metal cages under strict hygienic conditions. The rats were maintained on standard laboratory diet (15gm/rat/day) and fresh water ad libitum.

Chemicals: N-nitrosodiethylamine was purchased from Sigma Aldrich (3050 Spruce Street, Saint Louis, MO 63103, USA) and was dissolved in corn oil. Artichoke (evaporated aqueous extract from whole fruit) was provided as capsule from Western Pharmaceutical Industries, Egypt and dissolved in carboxy methyl cellulose.

Treatments: Animals were divided into four groups, ten rats each:
Group I: Negative control. Group II (experimental control for artichoke): artichoke was orally given at a dose of 300mg/kg bw, dissolved in carboxy methyl cellulose, daily for 14 weeks.
Group III: (hepatic fibrosis model group): From the beginning of the third week of the experiment rats were IP injected with DEN at the dose of 50mg/kg bw, once weekly dissolved in corn oil for development of hepatic fibrosis.
Group IV: (protective group): rats were firstly given artichoke (orally
via stomach gavage) at a dose of 300 mg/kg B wt, dissolved in carboxy methyl cellulose daily in the first two weeks prior to DEN injection and for 14 weeks. From the beginning of the third week of the experimental period, rats were injected I/P with 50mg/kg DEN in corn oil once weekly, 2 hours after artichoke treatment. At the end of 14th week of the experiment, all rats were anesthetized with ether. Blood was collected from retro-orbital venous plexus and serum was separated at 3000 rpm for biochemical parameters. Livers were removed rapidly and fixed in 10% formalin for histopathological and immunohistochemical studies and the other piece was freeze for measuring Redox state.

**Determination of liver enzymes:**
Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were determined colorimetrically. ALT and AST were determined using commercial kits provided by Randox, United Kingdom according to *Schmidt and Schmidt* (1963). ALP was determined using commercial kits provided by Biodiagnostic, Egypt according to *Belfield and Goldberg* (1971).

**Determination of Redox State:**
Diagnostic kits for colorimetric determination of redox state were commercially provided by Biodiagnostic, Egypt. Superoxide Dismutase (SOD) was assayed according to the method described by *Nishikimi et al* (1972), Glutathione (GSH) according to *Beutler et al* (1963), Catalase (CAT) according to *Aebi* (1984) and Malondialdehyde (MDA) according to *Satoh* (1978).

**Histopathological Examination:**
Tissue specimens from the liver of rats in different groups were collected and immediately fixed in 10% neutral buffered formalin. After proper fixation, the specimens were dehydrated in ethyl alcohol, cleared in xylol, embedded and casted in paraaffin. Thin paraaffin sections (4-5µ) thick were prepared and stained with routine stain hematoxylin and eosin and special stain (Masson’s trichrome) according to *Bancroff et al* (1990). Hepatic fibrosis was graded using semi-quantitative scoring system according to the method of *Ruwart et al* (1989).

**Immunohistochemical Study:**
The standard immunohistochemical methods were adopted according to *Eissa and Shoman,* (1998). Universal systems used were the biotin-streptavidin (BSA) system to visualize the markers (Hsu *et al.*, 1981). Diaminobenzidine (DAB) was used as a chromogen since it allows a permanent preparation. Hematoxylin counter stain was done.

**Statistical analysis:**
Results were represented as means ±SE. The difference between groups was assessed by one way
analysis of variance (ANOVA) using SPSS for windows (version 20).

Results
Biochemical analysis:
Rats treated with artichoke showed no significant difference in the serum ALT, AST and ALP activities compared to the control. While ALT, AST & ALP activities showed significant increase in DEN treated group (III) compared to control one. In protective artichoke group (IV) ALT, AST & ALP revealed significant increase compared to control one and significant decrease compared to DEN treated group.

Results of Redox State:
Rats treated with artichoke showed no significant difference in MDA level, SOD & CAT activities and GSH level. While hepatic MDA level showed significant increase in DEN treated group (III) compared to control one. In protective artichoke group (IV) MDA significantly increased comparing to the control and decreased significantly compared to DEN treated group. SOD, CAT activities and GSH level revealed significant decrease in DEN treated group (III) compared to the control. In protective artichoke (IV) a significant decrease was noticed compared to the control and a significant increase compared to DEN treated group.

Histopathological results:

Group I (normal control): Liver of the examined rats in this group were grossly normal in both color and consistency. Microscopically, the liver had normal hepatic cells, central veins and hepatic areas (plate 1-A). The sections stained by Masson trichrome stain revealed absence of fibrosis (plate 1-B). The semiquantitative scoring of fibrosis indicated grade 0. The immunohistochemical studies revealed normal α-SMA-positive stain around the central vein (plate 1-C). No intralobular immunoreactivity observed.

Group II (Artichoke control): Liver of rats in this group were grossly normal in both color and consistency. Microscopically, the liver had normal hepatic pattern of hepatic cells, central veins and hepatic areas as shown in control group. The sections stained by Masson trichrome stain revealed absence of fibrosis. The semiquantitative scoring of fibrosis indicated grade 0. The immunohistochemical studies revealed α-SMA-positive stain around the central veins. No intralobular immunoreactivity was observed.

Group III (fibrosis model group): Macroscopically, the liver was small, firm and pale with irregular coarse surface. Some cases showed adhesion of the hepatic capsule with the hepatic tissue. Few to many minute white foci were observed on hepatic surface. Microscopically,
the hepatocytes showed severe and diffuse degenerative changes mainly vacuolar degeneration. In some hepatic lobules, some hepatocytes showed mild and moderate degree of dysplasia. Dysplasia was represented by enlarged cells and enlarged irregular nuclei with dyschromasia, some hepatocytes had mitotic figures (plate 1-D). Focal areas of necrosis were also observed. In addition, extensive intralobular fibrosis in forms of both porto-portal and porto-central bridging fibrosis was observed (plate 1-E). Collagen precipitation was observed in hepatic areas (plate 1-F). Severe infiltration of hepatic areas with mononuclear cells mainly lymphocytes along with hyperplasia of bile ducts was also recorded (plate 2-A). Focal hepatitis was observed with various degrees of degeneration, necrosis and dysplasia of adjacent tissues (plate 2-B). Wall of some blood vessels showed thickening and hyalinization (plate 2-C). Fibrous tissue was extended around the hepatic cells (Periacinar fibrosis) (plate 1-D).

The sections stained by Masson trichrome stain revealed blue fibrous tissue in case of both porto-portal and porto-central bridging fibrosis (plate 1-E&F). The Semiquantitative-scoring method of fibrosis revealed the presence of grades-III and IV. The immunohistochemical staining showed increased expression of α-SMA positively stained brown and appeared as thick septa forming porto-portal and porto-central bridging fibrosis (plate 3-A&B). The Semiquantitative-scoring of fibrosis revealed the presence of grades- III and IV as thin and thick fibrous septa, respectively which resulted in either porto-portal or porto-central bridging fibrosis.

**Group IV** (Protective artichoke group): Macroscopically, the liver was apparently normal in size and Consistency with mild yellowish discoloration. Microscopically, the intensity of the degenerative and necrotic changes was mild when compared with group (IV). Fibrous tissue proliferation was observed around portal tracts along with mild mononuclear cell infiltration mainly lymphocytes (plate 3-C&D). The Masson trichrome stained sections showed mild fibrous tissue proliferation (plate 3-E). The semiquantitative scoring of fibrosis revealed grades I fibrosis with periportal and minimal intralobular fibrous septa (plate 3-F).
Table (1): Some serum biochemical parameters (Mean ± SE) in different groups of rats after 14 weeks:

<table>
<thead>
<tr>
<th>Groups</th>
<th>ALT U/L</th>
<th>AST U/L</th>
<th>ALP U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Cont.)</td>
<td>9.60^c</td>
<td>25.80^c</td>
<td>114.20^c</td>
</tr>
<tr>
<td></td>
<td>±0.97</td>
<td>±1.11</td>
<td>±1.11</td>
</tr>
<tr>
<td>II (Artichoke)</td>
<td>10.40^c</td>
<td>25.40^c</td>
<td>112.00^c</td>
</tr>
<tr>
<td></td>
<td>±0.97</td>
<td>±0.74</td>
<td>±0.94</td>
</tr>
<tr>
<td>III (DEN)</td>
<td>22.00^a</td>
<td>41.40^a</td>
<td>220.60^a</td>
</tr>
<tr>
<td></td>
<td>±0.83</td>
<td>±0.87</td>
<td>±1.32</td>
</tr>
<tr>
<td>IV (P. Artichoke)</td>
<td>15.00^b</td>
<td>35.20^b</td>
<td>182.00^b</td>
</tr>
<tr>
<td></td>
<td>±0.54</td>
<td>±0.96</td>
<td>±1.14</td>
</tr>
</tbody>
</table>

Cont. (control), DEN (diethylnitrosamine treatment), P. Artichoke (protective artichoke). Means with the same letter in the same column are non significant at p< 0.05.

Table (2): Some Tissue Oxidative Stress Markers (Mean ± SE) in different groups of rats after 14 weeks of the experiment:

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA µmol/g</th>
<th>SOD U/mg</th>
<th>CAT U/g</th>
<th>GSH mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Cont.)</td>
<td>0.85^c</td>
<td>14.65^a</td>
<td>78.79^a</td>
<td>64.06^a</td>
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<tr>
<td></td>
<td>±0.04</td>
<td>± 0.07</td>
<td>±0.29</td>
<td>±0.81</td>
</tr>
<tr>
<td>II (Artichoke)</td>
<td>0.84^c</td>
<td>14.71^a</td>
<td>79.46^a</td>
<td>62.53^a</td>
</tr>
<tr>
<td></td>
<td>±0.02</td>
<td>± 0.05</td>
<td>±0.24</td>
<td>±1.24</td>
</tr>
<tr>
<td>III(DEN)</td>
<td>2.01^a</td>
<td>11.79^c</td>
<td>73.15^c</td>
<td>28.41^c</td>
</tr>
<tr>
<td></td>
<td>±0.02</td>
<td>± 0.24</td>
<td>±0.30</td>
<td>±0.40</td>
</tr>
<tr>
<td>IV (P. Artichoke)</td>
<td>1.33^b</td>
<td>13.33^b</td>
<td>74.65^b</td>
<td>44.34^b</td>
</tr>
<tr>
<td></td>
<td>±0.07</td>
<td>± 0.04</td>
<td>±0.26</td>
<td>±0.74</td>
</tr>
</tbody>
</table>

Cont. (control), DEN (diethylnitrosamine treatment), P. Artichoke (protective artichoke). Means with the same letter in the same column are non significant at p< 0.05.
Plate (1) A,B&C: Liver of normal control group showing normal hepatic lobules, hepatic cords and Central veins (arrows). (A) H&E., (B) Masson trichrome stain and (C) normal immunoreactivity, limited to the wall of the central vein (arrows). (SMA , Hematoxylin counter stain & DAB as chromagen. X 40 . (D, E & F) Liver treated with DEN (D) showing moderate degree of dysplasia represented by enlarged irregular cells and nuclei with dyschromasia, and mitotic figures (arrow), (E): showing extensive intralobular fibrosis of both porto-portal and porto-central bridging fibrosis (grade IV fibrosis). (F): showing fibrosis of hepatic area with collagen precipitation (arrows) H&E X 40, X 10 & X40.
Plate (2): Liver treated with DEN (A) showing fibrosis of hepatic areas with collagen precipitation (arrows). (B) Showing focal hepatitis with various degrees of degeneration. (C) Showing thickened hyalinized vascular wall. H&E X40. (D) Showing periacinar fibrosis. H&E X40. (E&F) showing grade IV-interlobular bridging fibrosis of both porto-portal and porto-central bridging fibrosis, fibrous tissue invading the degenerated hepatocytes. Masson trichrome X10& X40.
Plate (3): (A&B), Liver treated with DEN showing positive immunoreactivity for α-SMA (brown) Porto-central bridging fibrosis and intra-acinar reaction of the delicate fibrous proliferation (arrow head). SMA, Hematoxlyin counter stain & DAB as chromagen. X40. C-F Liver treated with Artichoke and DEN (C) showing focal mild mononuclear cell infiltration mainly lymphocytes. (D) showing thin incomplete fibrous tissue proliferation around portal tracts (grade I fibrosis) H&E X 10. (E) showing incomplete fibrous tissue bridging (grade I). Masson trichrome. X 10. (F) showing early fibrous tissue proliferation ,a focal positive reactivity for α-SMA. SMA, hematoxylin as counter stain, DAB chromagen. X10.
**Discussion:**

Liver diseases such as fatty liver, hepatitis and liver fibrosis are important world health problems and usually lead to liver cirrhosis and hepatocellular carcinoma. The effective treatment with synthetic medical drugs had many side effects. For these reasons, developing drugs for liver diseases from plants used in traditional medicine may improve therapy and have products with reduced side effects in comparison to synthetic drugs. So, the present study demonstrated the protective effect of artichoke on DEN induced liver fibrosis as obtained by serum biochemical tests, oxidative stress and histopathology.

Serum AST, ALT and ALP are effective biomarkers in the diagnosis of hepatic damage. The results of the present study showed that DEN administration causes severe liver damage demonstrated by remarkable elevation of serum AST, ALT and ALP levels. This elevation may be attributed to the release of these enzymes from the cytoplasm into the blood circulation after rupture of the plasma membrane and cellular damage (Naik and Panda, 2007). Our results agree with Shaarawy et al (2009) who mentioned that the injection of DEN to rats lead to a marked elevation in AST, ALT and ALP. Histopathology of liver confirmed elevation of these enzymes as observed by severe degeneration and necrosis of hepatocytes.

Rats pretreated with artichoke extract showed significant decreases in plasma ALT, AST and ALP activities after DEN treatment compared to that of DEN -treated rats. This agreed with Mehmetcik et al (2008) who reported that in rats pretreated with artichoke extract, significant decreases in plasma ALT and AST activities were observed following CCl4 treatment as compared to CCl4-treated rats. The therapeutic activity of artichoke extract is probably due to phenolic structure of artichoke that is responsible for the free radical mediated processes inhibition (Hertog and Hollmann, 1998; Perez-Garcia et al., 2000). Histopathology of this group showed mild degeneration of hepatocytes and mild fibrosis with minimal to incomplete bridging (grade I fibrosis).

In DEN treated group, significant increase in MDA level was found compared with control group. This result agrees with Shaarawy et al (2009) who stated that there was a significant increase in the level of lipid peroxidation in the liver of rats treated with DEN.

In protective artichoke group the MDA showed significant decrease compared with DEN treated group. The result agreed with Mehmetcik et al (2008) who recorded that there was a significant decrease in hepatic MDA in CCL4 group pretreated with artichoke extract.
In the present experiment the activities of SOD, CAT and GSH level showed marked decrease in DEN treated group. This result agree with Shaarawy et al (2009) who reported that a significant decrease was noticed in SOD & CAT activity and GSH level in rats treated with DEN. 

In protective artichoke group the activities of SOD, CAT and GSH level showed significant increase compared with DEN treated group. These results agree partially with Mehmetcik et al (2008) who recorded that in rats pretreated with artichoke extract, significant increases GSH levels and GSH-Px activities increased without any change in other antioxidant system parameters.

The pathological and immunohistochemical staining of group II given artichoke was similar to group I (control) and showed nearly normal histological architecture. The liver of rats in group (III) was small, firm and pale due to the fibrous-tissue-proliferation in the portal areas and around the hepatic lobules. Microscopically the amounts of collagen fibers accumulated in the livers and fibrosis areas in the liver were enlarged significantly. Our results came in agreement with Nakazato et al (2010) who recorded that rats injected intraperitoneally with DEN, showed severe alteration with large fibrous bundles linking portal spaces and mild lymphocyte infiltration. The massive hepatic injury observed in the present study which manifested as diffuse degeneration, focal lymphocytic infiltrations and regenerative cellular changes, binucleation, nuclear enlargement are some of the regenerative cellular changes that agreed with Ahmed et al (2010) and Liu et al (2009). Treatment with DEN showed distinct alterations compared with untreated control rats, such as loss of lobular architecture, fibrosis, dysplasia and malignant nuclei , same results reported by Ramakrishnan et al (2006), and could be attributed to that Diethylnitrosamine is a powerful hepatocarcinogen. It is metabolized to reactive electrophilic reactants that alter the structure of DNA and forms alkyl DNA adducts (Yoshiji et al 1991). The increased expression of α-SMA-positively-HSC is stained brown, and appears as thick septa-forming porto-portal and porto-central-bridging-fibrosis. Our results are in concurrence with Gillibert et al (2004). The Artichoke significantly improved the gross picture and histopathological picture of the liver in group IV when compared with group III. Masson trichrome and α-SMA revealed that low grade fibrosis (grade I). The present results agreed with that of Mehmetcik et al (2008) who recorded that rats treated with artichoke showed mild to moderate leukocytic infiltration in the hepatic area with nearly normal hepatic architecture.
Finally, protective effect of artichoke is among the best characterized phytopharmaceutics. Their hepatoprotective, cholagogic, and hypolipemic properties have been documented in a wide range of experimental studies. The present and the previous findings have confirmed the traditional medical applications and importance of the extracts as recorded by Gebhardt (1998); Lupattelli et al (2004). Several studies defined the antioxidative and free radical scavenging potential of artichoke extracts as well as their particular components, i.e., caffeic acid, chlorogenic acid, cynarin, and luteolin. These bioactive components with antioxidative and antiproliferative activities are also constituents of the artichoke extract used in the present study. The antioxidant barriers of the artichoke extract’s constituents plays a role on the inhibition of ROS generation, ROS neutralization, or the induction of endogenous antioxidants as obtained by Juzyszyn et al (2008).

References:

Hertog MGL and Hollmann MCH. (1998): Potential health


الملخص العربي

"دراسات باثولوجية اكلينيكية و باثولوجية على استخدام الخرشوف كمادة واقية للكبد في الفئران البيضاء"

أجرت هذه الدراسة علي (40) فأر أبيض بمتوسط وزن 100-150 جم. تم تقسيمهم إلى أربعة مجموعات متساوية. مجموعة الأولى: تم تمثل المجموعة الضابطة. المجموعة الثانية: تم الحقن بالخرشوف (300 ملجم / كجم من وزن الجسم يوميا) لمدة 14 أسبوع. المجموعة الثالثة: تم الحقن بالنيتروزامين (50 ملجم / كجم من وزن الجسم أسبوعيا) ابتداءا من الأسبوع الثالث من التجربة ولمدة 12 أسبوع. المجموعة الرابعة: تم معالجتها بالخرشوف (300 ملجم / كجم من وزن الجسم يوميا) لمدة 14 أسبوع. وابتداءا من الأسبوع الثالث من التجربة تم حقن الفئران بالنيتروزامين (50 ملجم / كجم من وزن الجسم أسبوعيا). عند نهاية الأسبوع الرابع عشر تم تخدير الفئران وجميع الدم ثم فصل مصل الدم وجميع عينات الكبد. تم قياس انزيمات الكبد في مصل الدم. كما استخدمت عينات الكبد لقياس دلالات الإجهاد التأكسدي مثل مستوى المالونديالدهيد الدهون المختزل والسوبر أكسيدوموتاز والكتالاز.

وقد ظهرت نتائج الدراسة أن:

استخدام مادة النيتروزامين أدى إلى زيادة معنوية في نشاط إنزيمات الكبد والمالونديالدهيد. كما أدى إلى نقص معنوي في الجلوتاثيون ونشاط السوبر أكسيدوموتاز والكتالاز. وقد أظهر مستخلص الخرشوف تأثيره الواقي للKITO ضد الشوارد الحرارة ومؤشرات الإجهاد التأكسدي وتمثل في نقص مستوي إنزيمات الكبد والمالونديالدهيد وزيادة مستوي الإنزيمات الواقية من الأكسدة.