The Therapeutic Effects of Milk against Renal Toxicity in Rats

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Abstract
This study aimed to examine the therapeutic effect of milk thistle (MT) on the kidneys of diethylnitrosamine/ carbon tetrachloride (DEN/CCl₄) administration in rats. Twenty male albino rats weighing 130-170 gm were taken. It comprised of four groups (n=5): a control, MT treated, DEN/CCl₄ untreated and DEN/CCl₄ treated with MT. DEN was injected in a single dose (200 mg/kg, I.P.), then carbon tetrachloride (CCl₄) were injected I.P. for 2 weeks with 1ml/kg body weight. MT (500 mg/kg body weight) treatments were started 2 weeks after DEN/CCl₄ administration and continued for 8 weeks. Kidney was stained with H&E, PAS and Masson’s Trichrome. For scoring of the renal lesions, H&E sections were analyzed five morphological parameters including tubular necrosis, mononuclear cell infiltration, congested blood vessels, hyaline (tubular) cast and glomerular changes. Also, sections were immunostained with inducible nitric oxide synthase (iNOS) and analyzed using the ImageJ. The applied dose of DEN/CCl₄ caused histopathological alterations in the renal cortex such as tubular necrosis, swelling of the tubular epithelium, hyperplastic glomeruli and focal inflammatory cell infiltration. Moreover, there was a decrease in the PAS +ve material in the proximal convoluted tubules and an increase in collagen deposition in the interstitium and glomerular tufts. Treatment with MT after administration of DEN/CCl₄ attenuates the histological changes. A significant increase in the immunostaining intensity of (iNOS) of DEN/CCl₄ treated animals compared to the MT treated ones. The study concluded that MT post treatment prevented the histopathological changes caused by DEN/CCl₄. Therefore, MT can be used as an effective therapeutic agent against DEN/CCl₄-induced nephrotoxicity.

Key words: Milk thistle, Kidney, DEN/ CCl₄.

Introduction
The kidney is the major route for the excretion of metabolic products and toxins. In LPS-mediated endotoxaemia, renal tubular epithelial cells are exposed to high concentrations of LPS and these can...
adversely affect renal function and structure and may result in acute kidney injury (AKI). Basic studies in animal models of AKI have characterized changes in renal vasculature, tubules and interstitial tissue following acute insult.

The upregulation of chemokines, including tumour necrosis factor (TNF)-a and adhesion molecules, such as intercellular cell adhesion molecule (ICAM)-1, in the endothelium results in the infiltration of inflammatory cells from blood vessels into the renal interstitium. Interleukin (IL)-18 and IL-6 produced by the proximal tubule epithelial cells promote the activation and/or proliferation of these inflammatory cells.

The resultant vascular injury and increase in levels of reactive oxygen species, calcium and phospholipases lead to damage of the epithelial cytostructure. These changes also signal apoptosis and initiation of cell necrosis, which lead to tubular epithelial desquamation and obstruction. Although not all the pathological changes observed in animal models of ischaemia are seen in human renal tissue, morphological features of apoptosis, including karyopyknosis, cell membrane blebbing and apoptotic body formation, have been observed clinically in patients.

In addition, the central role of caspase 3, a cysteine protease and important terminal enzyme in apoptosis, has been reported to occur in humans with AK.

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In addition, the central role of caspase 3, a cysteine protease and important terminal enzyme in apoptosis, has been reported to occur in humans with AK. Kidneys are definitely liable to drugs and chemical toxins and actually; nephrotoxicity is the most public pathological disorder that leads to end-stage renal failure. 

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and Ryan, 1998). Several pathological injuries of the kidney have been associated with drug-induced nephron-toxicity (Sarang and Ameeta, 2001) that have been attributed to the free radicals and formation of reactive oxygen species (Ozbek, 2012). N-nitrosamines have been implicated in cellular injury due to the association of free radicals, like nitric oxide radical (Bansal et al., 2000). In foods, nitrosamines are formed from nitrates and secondary amines, which frequently arise in the form of proteins. DEN is widely distributed in the meat products, soybean, tobacco, fish products; cheese; and alcoholic beverages (Das et al., 2016). The cytotoxicity and carcinogenicity of the DEN administration were attributed to its ability to induce oxidative stress and toxicity (Barnes et al., 2010). Carbon tetrachloride (CCl₄) is metabolized by cytochrome P-450 in mitochondria and endoplasmic reticulum with the development of trichloromethyl peroxyl radicals a reactive oxidative free radical, which initiate lipid peroxidation (Sidana et al., 2011 and Hamouda & Shaban, 2015). Phytochemistry has been recorded many natural botanical medicines that have clinical significance and possess apoptotic, anti-proliferative, antioxidant and antimicrobial actions (Mahmoud et al., 2015). *Silybum marianum*, (milk thistle), is one of the non-toxic herbal medicine which used as hepatoprotective agent in many countries (Luper, 1998; Deep et al., 2012). It is a member of Asteraceae family. The main component of milk thistle is Flavolignans (silymarin), tyramine, histamine, gamma linoleic acid, essential oil (Ball and Kowdley, 2005). Silymarin is a lipophilic extract of the milk thistle seeds. It is composed of three isomers of flavonolignans (silybin, silydianin, and silychristin), and two flavonoids (taxifolin and quercetin) (Abenavoliet *et al*., 2010). Silibinin is the main constituent of silymarin, an extract from the seeds of milk thistle (*Silybum marianum*). Because silibinin has many pharmacological activities, extending its clinical use in the treatment of wider variety of diseases would be desirable (Yamasaki et al., 2017). Also, Elyasi et al. (2016) reported that Silymarin is a polyphenolic flavonoid extracted from the milk thistle that exhibited strong antioxidant and anti-inflammatory activities. This study was conducted to estimate the curative effect of milk thistle Ethanolic extract in the modulation of DEN/CCl₄-induced renal damages in male albino rats through histopathological and immunohistochemical methods.

**Materials and Methods**

**Animals**

This experiment was carried out on male albino rat, weighing 130-170 gm. rats were obtained from National Research Center, Dokki, Giza, Egypt. They were housed in well ventilated polystyrene cages at room temperature (24±2°C), in clean condition under natural light and dark schedule and were fed on standard laboratory diet (Corn, soya bean, calcium phosphate, limestone powder and table salt, El-Khams stars company, Misr El-
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gededa, Egypt). Food and water were given ad libitum. All animals received qualified care in agreement with National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, (2011).

Preparation of ethanolic extract of milk thistle seed (MT)

One kilograms of shade dried milk thistle, coarsely powdered, charged into aspirator bottles and allowed to soak in absolute ethanol (100%) for 72 hours at room temperature. The extracts were filtered, concentrated using rotary vacuum to get the solid mass. The residue yielded was 1.16% of milk thistle. The concentrate was dissolved in distilled water for oral administration. This was according to Osama et al., (2016).

Experimental design

A total of 20 adult male albino rats were divided into four groups of 5 animals each. Rats of group 1 (control group) received a single intraperitoneal (i.p.) injection of normal saline (2.5 ml/kg). Animals in group 2, DEN was dissolved in saline and injected in a single dose (200 mg/kg, I.P.) according to Pashmforoosh et al., (2015) then, CCL₄ was injected intraperitoneally for 2 weeks (3 times per week) with 1ml/kg body weight dissolved in olive oil of a 50 percent solution according to Sahreenet al., (2015). Group 3 were administered with MT (500 mg/kg BW). Dose of MT were selected on the basis of doses used by earlier researchers (Ghaffari et al., 2011). Plant extract treatments were started 2 weeks after DEN/CCl₄ administration and continued for 8 weeks (3 time/week). Animals in Group 4 (MT-supplemented-DEN/ CCL₄ group) were received the same doses as in group 2 then 2 weeks later the animals treated with MT extract for 8 weeks (500 mg/kg BW, 3 times per week).

Histopathological assessment.

At the end of the experiment, animals were sacrificed by cervical dislocation under anesthesia. Directly, experimental animals were dissected out and small pieces of kidney were fixed in 10% neutral buffered formalin, alcohol-dehydrated and paraffin-embedded. Embedded kidney tissues were sectioned at 4-6 μm thick. To evaluate the histological changes, sections were stained with Hematoxylin and Eosin (H&E), periodic acid-Schiff reagent and Masson's Trichrome. This was in accordance with (Suvarna et al., 2013).

Microscopic renal lesions were scored at a magnification of ×400. Five rats per group were used. Hematoxylin and Eosin sections were examined for analyzing of five morphological parameters including tubular necrosis, mononuclear cell infiltration, congested blood vessels, hyaline (tubular) cast and glomerular changes on a semi-quantitative score. Based on the intensity of tubular lesions as mentioned above, we scored from 1 to 4 while the score of zero was assigned to normal tubules without damage (Nematbakhsh et al., 2013)
**Immunohistochemical evaluation.**
Kidney sections were immunostained with inducible nitric oxide synthase (iNOS) as follow: Endogenous peroxidase of deparaffinized and rehydrated was blocked using hydrogen peroxide (3%) and, then subjected to 10mM citrate buffer (pH 6.0) for 10-20 minutes in a microwave. They were incubated in rabbit polyclonal iNOS antibody (Cat. # RB-9242-R7, Thermo Fisher Scientific, Fermonet, CA, USA) at room temperature. Binding of antibody– antigen reaction was detected by using avidin-biotin complex (ABC kit, Vector laboratories). Immunostaining reaction was labeled with diaminobenzidine (DAB) as a chromogen and counterstained with Mayer’s Hematoxylin. Negative control sections were prepared by the similar steps except they were incubated with the antibody diluent instead of primary antibody. The immunostaining intensity was investigated by light microscopy (Olympus DP25 Japan). For revealing of % area of iNOS immunostained reaction, kidney images were analyzed using the ImageJ software developed by the National Institute of Health (Bethesda, Maryland, USA).

**Statistical analysis**
Data were expressed as the mean ± S.E. Means were compared by one-way analysis of variance (ANOVA) followed by Duncan test, which was used to identify differences between groups. Differences were considered statistically significant at P< 0.05.

**Results**
Control kidney sections revealed normal histological structure of renal cortex with no histopathological injuries; the glomerular corpuscles with their tuft of blood capillaries and the proximal and distal convoluted tubules were observed (Fig.1A). Kidney sections of MT alone treatment were similar to the control group (Fig.1B). Following the administration of DEN/ CCl4 in group II, marked histopathological changes and cellular injury were evident (Fig.1C). This was demonstrated by either depletion of some glomeruli or existence of large sized and hyperplastic glomeruli. Disorganized glomerular capillary loops and accumulation of peri-glomerular pinkish fluid were observed. Also, sever tubular necrosis with swelling of the tubular epithelium and abolition of tubular lumen was observed. Lumens of the proximal tubules were occupied with hyaline casts (Fig.1C). Sections showed Perivascular and interstitial tubular edema with amorphous reddish material, focal mononuclear inflammatory cell infiltration and congested blood vessels (Fig.1C). Treatment with milk thistle after administration of DEN/ CCL4 attenuates the histopathological changes. Sections showed few distorted renal corpuscles with mild dilatation of the tubular lumen (Fig.1D). The proximal convoluted tubules appeared with their cuboidal lining. The recorded histopathological changes are summarized in table (1). The injury was found to be more severe
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In DEN/CCL$_4$ group than in treated and control group, but the degree of injury was less remarkable in DEN/CCL$_4$+ MT group compared with control group. Suggesting that milk thistle significantly protected the kidney from DEN/CCL$_4$ induced kidney injury.

Examination of kidney of control rat stained with PAS showed positive materials in the cortical tissues. Parietal and visceral walls of the Bowman's capsule, capillaries of the glomeruli, the basement membranes of the proximal and distal convoluted tubules and the brush borders of the proximal convoluted tubules exhibited strong positive reaction with PAS technique (Fig. 2 A). The Control kidney (Fig.2A) and treated with milk thistle (500 mg/kg) for 8 weeks (Fig. 2B) revealed normal PAS reaction in the glomeruli, Bowman's capsules, the basement membranes of the renal tubules and the brush borders of the proximal convoluted tubules. Microscopic observation of kidneys of rats treated with DEN/CCL$_4$ showed marked diminution in PAS positive material in the renal tubules (Fig. 2C). Kidney sections of the rats with co- treatment of the same dose of the milk thistle extract and DEN/CCL$_4$ exhibited no apparent changes in the polysaccharides in both of the renal corpuscles and tubules. The stain affinity was more or less as in the control group (Fig. 2D). These data showed a significant decrease (P< 0.05) in polysaccharides content in kidney DEN/CCL$_4$ treated group compared with control group. However, PAS stained sections of the cortex of the kidney in DEN/CCL$_4$ group treated with MT showed a significant strong PAS +ve reaction at brush borders of the proximal convoluted tubules as compared with DEN/CCL$_4$ treated group (Fig. 2 E).

Staining of renal cortex with Masson's Trichrome displayed dense deposition of blue stained collagen fibers in the interstitium around the renal tubules and in the capillary loops of renal corpuscles of DEN/CCL$_4$ treated kidney (Fig.3C). Sections of control (Fig.3A), milk thistle (Fig.3B) and treated rats with DEN/CCL$_4$ followed by treatment of MT for 8 weeks (Fig.3D) showed normal distribution of collagen fibers.

Immunohistochemical staining of renal cortex with inducible nitric oxide (iNOS) revealed intense immunoeexpression in the glomerular podocytes, tubular epithelium and mononuclear cells. This was more intense in the sections treated with DEN/CCL$_4$ (Fig. 4C) than the control (Fig. 4A), milk thistle (Fig. 4B) and treated group (Fig. 4D). Quantification of iNOS expression using semi-automated image analyses displayed a significant increase ofiNOS expression in kidney tissue of DEN/CCL$_4$ treated rats compared to control and a significant decrease in DEN/CCL$_4$ treated rats with MT compared to DEN/CCL$_4$-treated rats Fig (4E).

**Discussion**

Kidneys are fundamental in the removal of plentiful xenobiotics, including drugs and environmental chemicals, as well as
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endogenous metabolites (Leblanc, 2004). Reactive oxygen species (ROS) are generated during the detoxification of xenobiotics and drugs, and caused oxidative stress. Oxidative stress has been shown to be linked to kidney toxicity and diseases (Kumar et al., 2003; Shaban et al., 2003).

In the present study administration of milk thistle extract alone showed no change in kidney iNOS expression as well as PAS reaction and Collagen content compared to the control group. Also, treatment with milk thistle alone showed no histopathological changes as compared to control group. Milk thistle and its constituents have been safely used for centuries as herbal medicine mainly for the treatment of liver diseases (Barceloux, 2008 and Bhattacharya, 2011). Our results showed histopathological change in renal cortex after DEN/CCl₄ administration. There were signs of tubular necrosis and hyperplasia of the vascular component in glomerulus. These findings were in accordance with the observations of Jaramillo-Juárez et al., (2008) and Hamouda and Shaban (2015) in rats. DEN /CCl₄ treated rats showed the presence of apparently large renal corpuscles with mesangial cells proliferation. In agreement with these findings, Adewole et al., (2007) recorded that the hypertrophied renal glomer-uli and tubular necrosis was considered as pathological characteristics of DEN/CCl₄ intoxication. In an attempt to explain these findings Bartsch et al., (1989) stated that DEN, one of the most important environmental carcinogens has been suggested to cause the generation of ROS resulting in oxidative stress and cellular damage. DEN metabolized by cytochrome p450 generates extremely reactive free radical, and initiates lipid peroxidation of the plasmalemma of the endoplasmic reticulum causing chain reaction. This reactive oxygen species could induce oxidative damage in DNA, proteins and lipids (Archer, 1989). Also, cortical sections of DEN/CCl₄ group revealed the presence of intratubular hyaline cast as well as peritubular congestion and amorphous pinkish exudates. Acidophilic hyaline casts demonstrated in some tubular luminae of the DEN /CCl₄ group might represent cellular debris that underwent molecular changes. Cells and their debris that detached from the tubular basement membrane combined with proteins present in the tubular lumina resulting in cast formation (Sadek et al., 2013). Regarding PAS stain of kidney tissue, DEN/CCl₄ administration displayed significant decrease of PAS reaction in the apical border and basement membrane of renal tubules indicating their loss or desquamation. These results were in consistent with Ziyadeh and Goldfarb (1995) and Sadek et al., (2013) who mentioned that the partial and complete loss of brush border was due to change in the carbohydrate composition of the glycocalyx.

The current study investigated the immunohistochemical localization of iNOS in the renal cortex after DEN/CCl₄ administration. Veelken et al., (2000) stated that iNOS expression was low or undetectable in normal kidneys, whereas the substantial increase in the amounts
of iNOS in the glomeruli and the renal interstitium was associated with nephropathies. This might be due to an enhanced generation of superoxide radicals and hydrogen peroxide radicals that accelerated peroxidation of native membrane lipids. Peroxidation of the mitochondrial membrane led to a loss of cell integrity and increase in membrane permeability that contributes to cell death (Shaban et al., 2014 and Hamouda & Shaban, 2015). Treatment with ethanolic extract of milk thistle after DEN/CCl₄ administration showed marked decreases in the expression of iNOS as well as collagen deposition but showed significant increases in the PAS reaction as compared to the DEN/CCl₄ group, this result could be attributed to antioxidant, scavenging free radicals activity (Das et al., 2008 and Li et al., 2017). The therapeutically active constituents of milk thistle seeds are three isomeric flavonolignans namely silibinin (silybin), silychristin, and silidianin which were recorded as potent antioxidants (Bhattacharya, 2011). Silymarin treatment prevented tissue damage and considered as a potentially free radical scavenger in kidney injury induced in rats [Homsi et al., (2010) and Soto et al., (2010)]. The protective effect of silymarin appeared to depend on many properties such as; its ability to inhibit the absorption of toxins, preventing them from binding to the cell surface and inhibiting membrane transport systems (Brandon-Warner et al., 2012). Also, this might be owing to its activity against lipid peroxidation (Muriel et al., 1992). Renal fibrosis is an excessive accumulation of extracellular matrices such as collagen and fibronectins and considered the principal process involved in the progression of chronic kidney disease (Pradère et al., 2008). The current results showed, in addition to histo-pathological damage in kidney that DEN/CCl₄ treatment resulted in increased collagen deposition. Our data clearly revealed that milk thistle treatment diminished the collagen level and showed reduction in collagen deposition after 8 weeks. These results were most probably attributed to anti-fibrogenic effect of milk thistle. This was in accordance with Jia et al., (2001) who suggested that Silymarin suppresses expression of profibrogenic procollagen α1 most likely via down regulation of transforming growth factor (TGFβ1) mRNA.

Conclusion
It can be concluded that ethanolic extract of milk thistle has a significant therapeutic effect against nephrotoxicity induced by DEN/CCl₄ in rats, which may be due to its free radical scavenging effect and its ability to increase antioxidant activity. As milk thistle might help cells to withstand and overcome the stress response via restoring histopato pathological changes and down regulation of iNOS in renal cortex.

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Table (1): Histopathological changes in the kidneys of experimental induced injury rats and treated ones with milk thistle extract.

<table>
<thead>
<tr>
<th>Groups: Control, Milk thistle treated group (MT), Diethylnitrosamine and Carbon tetrachloride (DEN/CCl4), Diethylnitrosamine and Carbon tetrachloride treated with milk thistle (DEN/CCl4+MT).</th>
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<tbody>
<tr>
<td>Groups: Control, Milk thistle treated group (MT), Diethylnitrosamine and Carbon tetrachloride (DEN/CCl4), Diethylnitrosamine and Carbon tetrachloride treated with milk thistle (DEN/CCl4+MT).</td>
</tr>
<tr>
<td>tubular necrosis</td>
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<tr>
<td>------------------</td>
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<tr>
<td>Control</td>
</tr>
<tr>
<td>MT</td>
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<tr>
<td>DEN/CCl4</td>
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<td>DEN/CCl4+MT</td>
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<table>
<thead>
<tr>
<th>Groups</th>
<th>tubular necrosis</th>
<th>mononuclear cell infiltration</th>
<th>congested blood vessels</th>
<th>tubular cast</th>
<th>glomerular changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>MT</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>DEN/CCl₄</td>
<td>2.00±0.21ᵃ</td>
<td>1.08±0.49ᵃ</td>
<td>1.70±0.30ᵃ</td>
<td>1.27±0.14ᵃ</td>
<td>1.45±0.10ᵃ</td>
</tr>
<tr>
<td>DEN/CCl₄+ MT</td>
<td>1.00±0.17ᵇ</td>
<td>0.73±0.19ᵇ</td>
<td>0.30±0.00ᵇ</td>
<td>0.79±0.09ᵇ</td>
<td>0.63±0.08ᵇ</td>
</tr>
</tbody>
</table>

Groups: Control, Milk thistle treated group (MT), Diethylnitrosamine and Carbon tetrachloride (DEN/CCl₄), Diethylnitrosamine and Carbon tetrachloride treated with milk thistle (DEN/CCl₄+ MT). Data presented as mean ± S.E (n=5) a’ compared with control group and ‘b’ compared with DEN/CCl₄ group. Values were statistically significant at p≤ 0.05.
**Fig (1):** Representative histological photomicrographs of Hematoxylin and Eosin-stained renal cortex showing (A) control, having normal structure of renal glomeruli and tubules, (B) milk thistle-treated group, similar to control (C) DEN/CCI₄-treated group showing apparently large sized renal corpuscle with proliferation of mesangial cells and glomerular hyperplasia (head arrow). Renal tubules displayed cloudy swelling with narrow lumen, acidophilic cytoplasm and pyknotic nuclei. Congested blood vessels (co) and intratubular cellular casts (arrow), and (D) DEN/CCI₄-MT treated group with mild dilatation of renal tubules (arrow). Scale bar 50 µm.
Fig (2): Histological sections of the cortical tissue of the kidney of shows the polysaccharides inclusions. (A) Control group showing PAS positive reaction in brush border of the cells lining the PCTs and in the glomerular blood capillaries (arrow). (B) MT-treated group showing PAS positive reaction in mesangial matrix of renal corpuscle and the brush borders of tubular epithelial cells in some renal tubules. C) Rat treated with DEN/CCl₄ showing marked decrease in PAS reaction. The glomerulus showed moderate PAS reaction. The degenerative cells and destructive brush borders showed faint stain affinity, D) rat treated with DEN/CCl₄ followed by treatment of MT for 8 weeks shows normal distribution of polysaccharides in the renal corpuscle (*) and renal tubules. Scale bar 100 µm.(e) Shows semi quantitative analysis of PAS reaction results in kidney. Data presented as mean ± S.E (n=5) a’ compared with control group and ‘b’ compared with DEN/CCl₄ group. Values were statistically significant at p≤ 0.05.
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Fig (3): photomicrographs of renal cortex stained with Masson's Trichrome. DEN/CCL₄ treated kidney (C) showed blue stained collagen fibers in the interstitium around the renal tubules (arrow) and in the renal tuft of renal corpuscles (arrowhead). Sections of control (A), milk thistle (B) and treated group (D) rat treated with DEN/CCL₄ followed by treatment of MT for 8 weeks showed normal distribution of collagen fibers. Scale bar 100 µm.
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**Fig (4):** Effect of Milk thistle (MT) extract after the induction of kidney injury (8 weeks) on the immunostaining of inducible nitric oxide synthase (iNOS) of DEN/CCl₄ treated renal cortex. Localization of iNOS immunoreactivity in the kidney of (A) control group, (B) MT-treated group, (C) DEN/CCl₄ -treated group, a positive immune-reaction of iNOS was found in apparently large renal corpuscles, degenerated tubules and in the interstitial leukocytic.
infiltration. (D) Associated DEN/CCl₄ with MT-treated group, the administration of Milk thistle resulted in a negative immune-reaction of iNOS in all the different structural components of renal cortex. (e) Show percent area of iNOS immunohistochemical staining of renal cortex. Data presented as mean ± S.E (n=5) a’ compared with control group and ‘b’ compared with DEN/CCl₄ group. Values were statistically significant at p≤ 0.05.