Protective Effect of Rosemary (*Rosmarinus Officinalis*) Leaves Extract on Carbon Tetrachloride - Induced Nephrotoxicity in Albino Rats

Saber A. Sakr[1] and Hawazen A. Lamfon[2]

[1.] Zoology Department, Faculty of Science, Menoufia University, Egypt
[2.] Department of Biology, Faculty of Applied Sciences, Umm Al-Qura University, Makkah, Saudi Arabia
sabsak@yahoo.com

Abstract: Carbon tetrachloride (CCl_4) is an environmental pollutant that showed toxicity in different organs. Exposure to CCl_4 is known to induce the formation of reactive oxygen species (ROS). Rosemary *(Rosmarinus officinalis*) is a herb commonly used as spice and flavoring agents in food processing and is useful in treatment of many diseases. The purpose of present study was to investigate the protective role of rosemary on CCl_4-induced renal damage. Treating rats with 1.0ml /kg body weight of 10% CCl_4 twice a week for 6 weeks induced many histological changes in the kidney cortex .The renal tubules lost their characteristic appearance and their lining epithelial cells were degenerated. The glomeruli were atrophied and the renal blood vessels were congested. The intertubular spaces were infiltrated by inflammatory leucocytic cells. An increase in interstitial expression of α-SMA was observed compared with control group. CCl_4 also caused marked elevation in serum creatinine and urea. Treating animals with CCl_4 and aqueous extract of rosemary led to an improvement, in both biochemical and histopathological pictures. It is concluded that rosemary extract had a protective effect against kidney injury induced by CCl_4 and this effect may be attributed to its antioxidant activity.

[Saber A. Sakr and Hawazen A. Lamfon. **Protective Effect of Rosemary (*Rosmarinus Officinalis*) Leaves Extract on Carbon Tetrachloride -Induced Nephrotoxicity in Albino Rats.** Life Science Journal 2012; 9(3):779-785]. (ISSN: 1097-8135). http://www.lifesciencesite.com.112

Keywords: CCl_4, Rosemary, Nephrotoxicity, Creatinne, urea, α-SMA

1. Introduction

Natural antioxidants strengthen the endogenous antioxidant defenses from reactive oxygen species (ROS) and restore the optimal balance by neutralizing the reactive species. They are gaining immense importance by virtue of their critical role in disease prevention. Rosemary *(Rosmarinus officinalis*) is a herb commonly used as spice and flavoring agents in food processing (Ho *et al.,*1994). Rosemary composed of dried leaves and flowers constitutes a particularly interesting source of biologically active phytochemicals as it contains a variety of phenolic compounds including carnosol, carnosic acid, rosmanol, 7-methyl-epirosmanol, isorosmanol, rosmadial and caffeic acid, with substantial *in vitro* antioxidant activity (Aruoma *et al.,* 1992). Leaves of rosemary possess a variety of bioactivities including antitumour (Singletary *et al.,* 1996) and anti-inflammatory actions (Altinier *et al.,* 2007). It is also useful in treatment or prevention of bronchial asthma, spasmogenic disorders, peptic ulcer, inflammatory diseases (Al-Sereiti *et al.,* 1999), hepatotoxicity, atherosclerosis biliary upsets, as well as for tension headache, renal colic ,heart disease, and poor sperm motility **(**Rampart *et al.,* 1986; Al-Sereiti *et al.,* 1999).The antioxidant potential of rosemary and its constituents has predominantly been derived from *in vitro* and *in vivo* studies (Richerimer *et al.,* 1996 ; Plouzek *et al.,* 1999). When rosemary extract was supplemented to chicken, it slows down effectively the lipid peroxidation (Serdaroglu and Yildiz-Trup, 2004). Lo *et al.* (2002) reported that carnosol, a naturally occurring polyphenol found in rosemary leaves, showed a potent antioxidative activity against α-diphenyl-B-picryldrazyl free radicals produced from Fenton reaction

Carbon tetrachloride (CCl_4) intoxication in animals is an experimental model that mimics oxidative stress in many pathophysiological situations (Mc Gregor and Lang, 1996). Carbon tetrachloride toxicity has resulted in many cases of poisoning by inhalation, ingestion or absorption. Prolonged exposure to carbon tetrachloride induced histopathological features such as inflammatory leucocytic infiltration, necrosis, fibrosis, cirrhosis and sometimes may lead to tumors (Qiu *et al.,* 2005). Jaramillo-Juárez *et al.* (2008) found that poisoning by CCl_4 induced toxic injury to both liver and kidney. Hepatic damage may be overshadowed by acute renal tubular necrosis, leading to renal oliguric of many species. Various studies demonstrated that CCl_4 intoxication caused free radical generation in many tissues such as liver, kidney, heart, lung, brain and blood (Dashti *et al.,* 1989). Ogeturk *et al.* (2005) reported that exposure to CCl_4 causes acute and chronic renal injuries. In addition, report on various documented case studies established that CCl_4 produces renal diseases in human (Ruprah *et al.,* 1985). The aim of the present study was to examine the protective effect of aqueous extract of rosemary in kidney in carbon tetrachloride intoxicated rats.

2. Materials and Methods

2.1. Preparation of rosemary extract

Extraction of rosemary was carried out according to the method of Dorman *et al.* (2003). Briefly, 50 g fine powdered herb were mixed with 500 ml distilled water in a quick fit flask round-bottom flask which connected to a hydrodistillation apparatus and the water was left to boil slowly for 120 minutes. The water from the flask was removed and another 300 ml of fresh distilled water were added and was boiled another 60 minutes. Water fractions were combined and filtered through qualitative Whatman filter. The filtrate was then subjected to lyopholyzation process through freeze drier under pressure, 0.1 to 0.5 mbar and temperature -35 to -41°C conditions. The dry extract was stored at 4°C until used. The used dose was 220mg/kg body weight.

2.2. Animals and treatments

Male albino Wistar rats weighting 100 ± 5 g were kept in the laboratory under constant conditions of temperature (24 ± 2 ^{o}C) for at least one week before and through the experimental work, being maintained on a standard diet and water were available *ad-libitum*. The animals were maintained in accordance with the guidelines prescribed by the Faculty of Science and the study was approved by the Animal Ethics Committee of the University of Menoufia, Egypt. The experimental rats were divided into four groups:

Group1: Animals were fed on the standard diet and were served as control group.

Group2: Rats were injected intraperitonealy with 1.0ml /kg b.w of 10% CCl_4 dissolved in olive oil twice a week for six weeks (Sakr *et al.*, 2007).

Group3: Animals were orally given 220mg/kg b.w. aqueous extract of rosemary**,** twice weekly for six weeks.

Group4: Rats were injected with CCl_4 followed by oral administration of rosemary extract, twice a week for six weeks.

2.3.Histological and immunohistochemical examinations

The treated animals and their controls were sacrificed by decapitation after 4 and 6 weeks of treatment. Their kidneys were removed and fixed in 10% neutral formalin. Fixed materials were embedded in paraffin wax and sections of 5 micrometer thickness were cut. Slides were stained with haematoxylin and eosin for histological examination. For immunohistochemical localization of α-SMA, fixed wax sections were stained using the avidin-biotin peroxidase method. Formalin fixed paraffin-embedded tissue sections were deparaffinized and endogenous peroxidase activity was blocked with PBS, 0.3% H_2O_2, and 10% methanol for 45 min. To prevent nonspecific binding, the sections were incubated for 60 min in PBS containing 0.3% Triton X-100, 1% BSA, 4% goat serum (GS), and 4% horse serum (block solution). The sections were then incubated overnight at 4 °C with mouse monoclonal α-SMA primary antibody: Actin, Smooth Ab -1(1A4) mouse MAb MS- 113 -PO (1:100; lot: 113P101, Neo Markers Fremont,CA, USA. Thereafter, the sections were incubated for 1 h with Biotinylated Horse Anti-Mouse/Rabbit IgG secondary antibody (Vector Laboratories).Sections were then incubated with avidin–biotin-conjugated peroxidase or 45 min. Finally, the sections were washed and stained with 3,3-diaminobenzidine tetrahydrochloride (DAB) (Sigma) containing 0.01% H_2O_2 in 0.05M Tris-buffered saline (pH 7.6) for 3–5 min. After the enzyme reaction, the sections were washed in tap water, counterstained with hematoxylin then dehydrated in alcohol, cleared in xylene, and mounted in DPX (Merck, Darmstadt, Germany). Area of α-SMA positive staining was assessed in predetermined high power field (40X) of the cortex (10 fields) then was captured by a digital camera (Kawai *et al.*, 2009).

2.4. Biochemical assays

For biochemical study sera were obtained by centrifugation of the blood samples and stored at 2O°C until assayed for the biochemical parameters. Creatinine and urea were estimated using the methods of Henry (1974) and Patton and Crouch (1977), respectively.

2.5. Statistical analysis:

The results were expressed as mean ± SD of different groups. The differences between the mean values were evaluated by ANOVA followed by Student's "t" test using Minitab 12 computer program (Minitab Inc., State Collage, PA). $P<0.05$ values were considered significant.

3. Results

3.1. Histological results

Histological examination of the kidney of control rat or rosemary- treated ones revealed entirely normal histological features, illustrated in figure (1a). The administration of CCl_4 caused significant histological damage to the kidneys, especially to the renal cortex. Examination of the kidney sections of animals after treatment with CCl_4 for 4 weeks, revealed enlargement and congestion of renal blood vessels (Fig.1b). Most of the renal tubules were damaged and lost their characteristic appearance and their lining epithelial cells became undistinguished. Intertubular leucocytic infiltrations were observed (Fig.1c). Marked alterations were observed after 6 weeks of treatment with CCl_4. A number of glomerular capillaries were suffering from severe signs of glomerular congestion, while others were completely damaged. The epithelial lining cells of the glomerular capillaries and of the Bowman's capsules were desquamated into the urinary spaces in the form of granular eosinophilic materials

(Fig.2a). The epithelial lining cells of the renal tubules were revealed nuclear pyknosis and variable forms of cellular rupture and damage. Tubular casts and flocculent materials were also noticed in the lumina of many tubules (Fig.2b). Treating animals with CCl_4 and rosemary revealed an improvement in the histological appearance of the kidney. Most of the renal tubules appeared normal but few tubules were damaged (Fig.2c). Comparison of changes in renal structures of different groups is summarized in table 1.

3.2. Immunohistochemical results

Kidney of control or rosemary-treated rats showed expression of α-SMA in the smooth muscle cells of renal arterioles (Fig.3a). An expression of α-SMA positive fibroblastic cells was recorded in the kidneys of CCl_4-treated rats (Fig.3b). Treatment with rosemary reduced interstitial expression of α SMA (Fig.3 c). Data in figure 4 showed that the percentage of α-SMA positive staining area was significantly ($P<0.05$) decrease in CCl_4- treated rats and the percentage decreased after treatment with rosemary.

3.3. Biochemical results

Treating animals with CCl_4 caused significant elevation in creatinine in the sera. On the other hand, a significant decrease was recorded after treatment with CCl_4 and rosemary (Fig.5). Similarly, blood urea exhibited a significant increase in the treated animals. Co-administration of rosemary lead to a decrease of blood urea (Fig.6).No significant change was recorded in values of creatinine and urea between rosemary and control group.

Table 1 Quantitative assessment of renal histological changes in different animal groups.

Animal Group	Tubular degeneration		Tubular cast		Leucocytic infiltrations		Glomeruli Atrophy	
	3 w	6 w	3 w	6 w	3 w	6 w	3 w	6 w
Control	-	-	-	-	-	+	-	-
Rosemary	-	-	-	-	+	+	-	-
CCl_4	+ +	+ + +	+ +	+ + +	+ + +	+ + +	+ +	+ + +
CCl_4+ rosemary	+	+	+	+	+ +	+	+	+

+ Mild (5–10% severity), + + Moderate (10–25% severity), + + +Severe (25–50% severity)

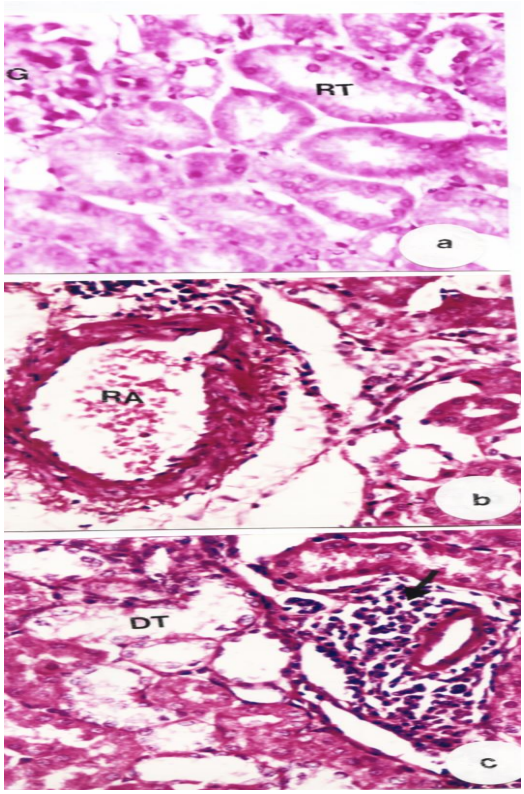

Fig.1. Sections in the kidney cortex of (a) control rat showing glomeruli (G) and renal tubules (RT); (b-c) 4 weeks after CCl_4 treatment showing enlarged and congested renal vein (RV), leucocytic infiltrations (arrow) and degenerated tubules (DT), (X300)

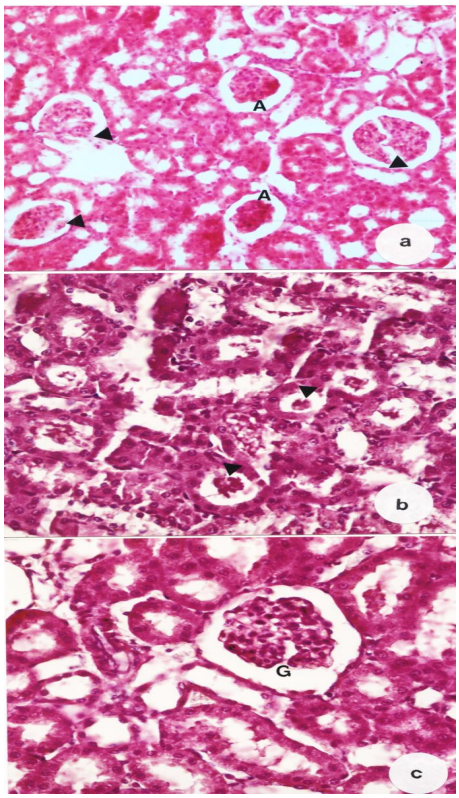

Fig.2. (a) Kidney cortex 6 weeks after CCl_4 treatment showing atrophied (A) and fragmented (arrow heads) glomeruli (X120), (b) proteinaceous casts in the lumen of renal tubules (arrow head) (X300), (c) after treatment with CCL_4 + rosemary showing normal renal tubules and glomeruli (G) (X300).

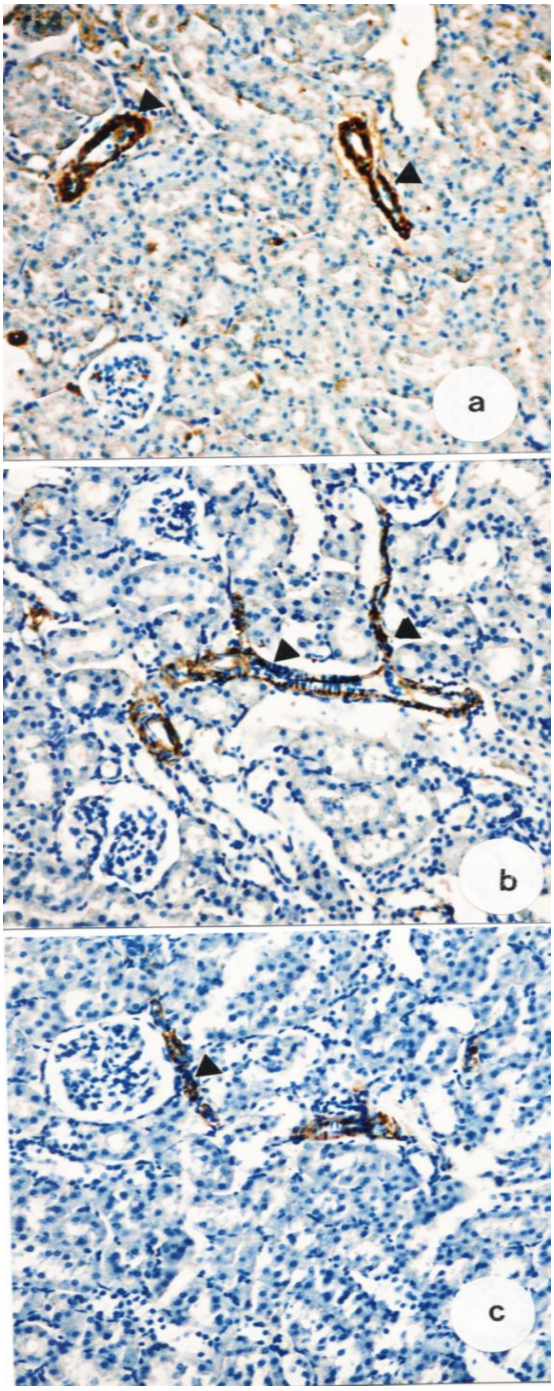

Fig.3. Immunohistochemical staining for *α*-SMA; (a) control kidney showing expression of *α*-SMA in renal arteriols (arrow head); (b) six weeks after treatment with CCl_4, showing increase in expression of *α*-SMA positive fibroblastic cells (arrow heads); (c) after treatment with CCl_4 and rosemary showing decrease of *α*-SMA expression (X 120).

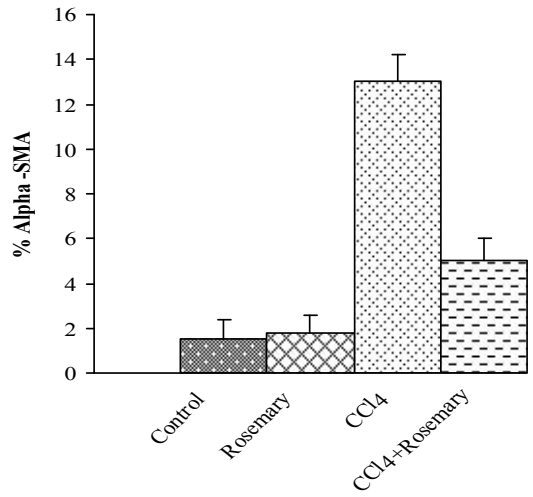

Fig.4. *%* *α*-SMA expression in different animal groups

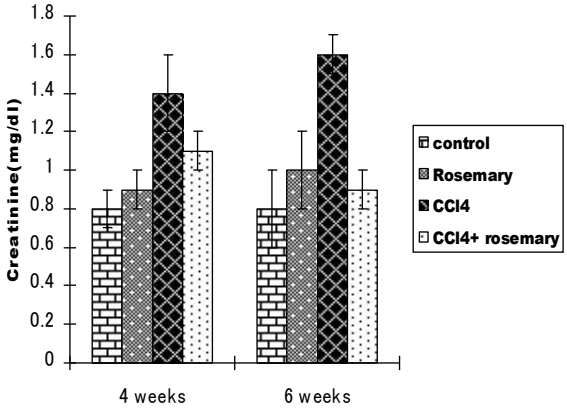

Fig.5. Change in creatinine in different animal groups.

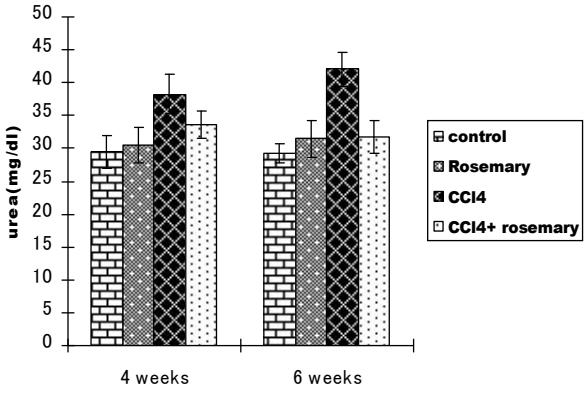

Fig.6. Change in urea in different animal groups.

4. Discussion

The elevation in creatinine and blood urea, and the observed histopathological alterations recorded in this work indicated that CCl_4 caused renal toxicity in rats. Ozturk *et al.* (2003) recorded similar histopathological alterations in rats kidney treated with CCl4 characterized by tubular epithelial cells alterations including vacuolization, atrophy, detachment of epithelial cells and tubular necrosis. With these histopathological changes, the capacity of tubular absorption may have been altered and functional overloading of nephrons with subsequent renal dysfunction was observed (Khan *et al.*, 2010). In addition to its hepatic toxicity, a number of reports clearly demonstrated that CCl_4 also causes disorders in kidneys, lungs, testes as well as in blood (Ahmad *et al.*, 1987; Ozturk *et al.*, 2003). Ogeturk *et al.* (2005) reported that exposure to this solvent causes acute and chronic renal injuries. Ruprah *et al.* (1985) established that CCl_4 produces renal diseases in human.

It was reported that CCl_4 metabolized by cytochrome p- 450 generates a highly reactive free radical, and initiates lipid peroxidation of the cell membrane of the endoplasmic reticulum and causes a chain reaction. These reactive oxygen species can cause oxidative damage in DNA, proteins and lipids (Melin *et al.*, 2000). Various studies have demonstrated that CCl_4 causes free radical generation in many tissues including kidney. Olagunjua *et al.* (2009) suggested a role for reactive oxygen metabolites as one of the postulated mechanisms in the pathogenesis of CCl_4 nephrotoxicity. Noguchi *et al.* (1982) reported that CCl_4 resulted in enhanced generation of trichloromethylperoxyl radical hydrogen peroxide in cultured hepatocytes as well as mesangial cells. *In vitro* and *in vivo* studies indicate that CCl_4 enhances lipid peroxidation, reduces renal microsomal NADPH cytochrome P450, and renal reduced/oxidiced glutathione ratio (GSH/GSSG) in kidney cortex as well as renal microsomes and mitochondria (Rungby and Ernst, 1992).

Concerning the immunohistochemical results, an increase in expression of □-SMA was recorded in kidneys of CCl_4-treated rats. α-SMA expression is a typical molecular marker of myofibroblasts in many nephropathies (Kramer *et al.*, 2009). Renal fibrosis is the principal process involved in the progression of chronic kidney disease (Pradère *et al.*, 2008), ureteral obstruction, malignant hypertension, severe diabetic condition or chronic exposure to heavy metals (Cohen, 1995). The development of renal fibrosis involves the progressive appearance of glomerulosclerosis, tubulointerstitial fibrosis and changes in renal vasculature, and at a molecular level, fibrosis can be defined as an excessive accumulation of extracellular matrix such as collagen and fibronectins (Al-Bayati *et al.*, 2002).

The current study revealed that rosemary aqueous extract alleviated the renal toxicity of CCl_4. This was manifested by normal appearance of kidney tissues and decreased levels of creatinine and urea. Similarly, Ahmed and Abdella (2010) reported that rosemary prevented histopathological lesions and oxidative stress induced by doxorubicin in liver, kidney and heart of mice. Rosemary was also found to have a therapeutic potential in treatment or prevention of inflammatory diseases hepatotoxicity, renal toxicity and heart diseases (Babu, 1996, Válenzuela *et al.*, 2004).

Abdel-Wahhab *et al.* (2011) reported that administration of rats with rosemary extract alleviated the deleterious effect of CCl_4 on liver. They added that rosemary may act as a co-factor in the synthesis of biological endogenous antioxidant material such as glutathione-s-transferase and quinine reductase. Interstitial fibrosis was decreased in kidney of rats treated with CCl_4 and rosemary as indicated by decrease of expression of α-SMA. In agreement with this result, Yahuaca *et al.* (2005) reported the protective effect of rosemary on liver fibrosis and cirrhosis induced by CCl_4.

Rosemary is rich in phytochemical derivatives such as triterpenes, flavonoids or polyphenols. Many studies reported that the preventive effects of rosemary and its extracts are attributed to its antioxidant activity. Schwarz *et al.* (1992) and Zeng and Wang (2001) reported that carnosol, rosmanol and epirosmanol phenolic diterpenes of rosemary inhibit lipid peroxidation. Ursolic acid, a constant constituent of *Rosmarinus officinalis* extracts, has been shown to have antioxidant and anticarcinogenic properties (Huang *et al.*, 1994). Rosmarinic acid exhibits antioxidant and antiinflammatory effects (Halliwell, 1996). Rosemary extracts are able to donate electrons to reactive radicals, converting them to more stable and on reactive species, therefore preventing them from reaching biomolecules, such as lipoproteins, polyunsaturated fatty acids, DNA, amino acids, proteins and sugars, in susceptible biological systems. Also, it was concluded that rosemary extracts have a high scavenging capacity of different types of reactive oxygen and nitrogen species, mostly free radicals, is thought to be one of the main mechanisms of the antioxidant action exhibited by phenolic phytochemicals (Moreno *et al.*, 2006).

In conclusion, the present results showed that rosemary aqueous extract alleviates the nephrotoxicity induced by CCl_4 in albino rats. This effect of rosemary may be attributed to the antioxidative activity of one or more of its constituents.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Corresponding author
Saber A. S
Zoology Department, Faculty of Science, Menoufia University, Egypt
sabsak@yahoo.com

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