

A Study on the Antioxidant Activity of Rosmarinic Acid Against Carbon Tetrachloride-Induced Liver Toxicity in Adult Male Albino Rats

Estudio sobre la Actividad Antioxidante del Ácido Rosmarínico Contra la Toxicidad Hepática Inducida por Tetracloruro de Carbono en Ratas Albinas Macho Adultas

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SUMMARY: Carbon tetrachloride (CCl₄) is a manufactured chemical and does not occur naturally in the environment. CCl₄ is a clear liquid that evaporates very easily. It has a sweet odor. CCl₄ is toxic to the mammalian liver and is hepatocarcinogenic in both rats and mice. Rosemary (*Rosmarinus Officinalis*) is commonly used as a spice and flavoring agent in food processing. It is known for its antioxidant properties. The present study aims to investigate the antioxidant activity of rosmarinic acid (RA) on CCl₄-induced liver toxicity in adult male albino rats. Forty adult male albino rats were divided into 4 groups with 10 rats in each group. Group I (control group). Group II animals received RA at a dose of 50 mg/kg/day by oral gavage for 4 weeks. Group III animals received CCl₄ intraperitoneally at a dose of 3ml/kg twice weekly for 4 weeks. Group IV animals received CCl₄ Plus RA. At the end of the experiment, liver specimens are processed for histological, immunohistochemical, EM and biochemical studies. Administration of RA decreased the elevated serum liver enzymes (AST, ALT, and ALP), elevated MDA level and immunoexpression of the proapoptotic protein (Bax) induced by CCl₄. It increased reduced glutathione (GSH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and immunoexpression of the antiapoptotic protein (Bcl2). It also improved the histological and ultrastructural changes induced by CCl₄. It appears that Rosmarinic acid has protective effects against CCl₄-induced hepatotoxicity as indicated by biochemical, histological, immunohistochemical and ultrastructural results.

KEY WORDS: Carbon tetrachloride; Liver toxicity; Acid; Oxidative stress; EM.

INTRODUCTION

Exposure to toxic chemicals, environmental pollutants and drugs can cause cellular injuries through metabolic activation of reactive oxygen species (ROS) (Szymonik-Lesiuk *et al.*, 2003). Liver is the main organ involved in generation of reactive oxygen species (ROS) induced by various drugs and chemicals (Alric *et al.*, 2000). Carbon tetrachloride (CCl₄) is a well-known hepatotoxic industrial solvent (Kim *et al.*, 2010). Liver in particular is susceptible to oxidative stress due to direct release of CCl₄ metabolites and cytokines, which cause inflammatory response (Khan *et al.*, 2012).

Liver damage induced by CCl₄ is a commonly used model for the screening of hepatoprotective drugs. The acute hepatotoxicity of free radicals causes oxidative stress and membrane damage. These free radicals cause lipid peroxidation which results in hepatocellular damage and enhances formation of inflamed tissues (Shrivastava & Gilhotra, 2017). The rise in serum levels of AST, ALT and cholesterol has been attributed to the damaged structural integrity of the liver, because they are cytoplasmic in location and released into circulation after cellular damages (Dhiman *et al.*, 2012; Shrivastava & Gilhotra).

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Rosmarinic acid (a-o-caffeoyl-3,4-dihydroxyphenyl lactic acid) is a primary constituent of traditional oriental and Chinese herbal medicine which is obtained from many medicinal plants such as species of Boraginaceae and Lamiaceae. Rosmarinic acid possesses a wide spectrum of pharmacological properties and confers health benefits such as antioxidation, anti-inflammation, anti-tumor, anti-bacteria, anti-virus, immunomodulation, neuroprotection, and nephroprotection (Cao *et al.*, 2016).

This work was therefore performed to evaluate the potential antioxidant activity of rosmarinic acid on CCl₄-induced liver toxicity in adult male albino rats.

MATERIAL AND METHOD

Animals. The present study was carried out on 40 healthy adult male albino rats weighing from 200–250 g. They were purchased from the animal house of Assiut Faculty of Medicine, Assiut University, Egypt. The rats were housed in polypropylene cages under standard lightening in a temperature-controlled room (25 ± 2°C) and had free access to laboratory food and water throughout the experiment. They were acclimatized to their environment for at least two weeks before starting the experiment. This study was conducted in strict accordance with the recommendations of the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The research protocol with animal experimentation was approved by the local Institutional Animal Ethical Committee of Faculty of Medicine, NBU, KSA. All surgery was performed under sodium pentobarbital anesthesia and every effort was made to minimize suffering.

Experimental design. After acclimatization period, rats were randomly divided into four groups (ten rats in each) as follows:

Group I (control group): Received injection of olive oil (vehicle) intraperitoneally through the experimental period.

Group II: Received rosmarinic acid dissolved in DMSO (dimethyl sulfoxide) by oral gavage (Sigma-Aldrich Chemical Co. St. Louis, MO, USA) at a dose of 50 mg/kg/day for 4 weeks (Rocha *et al.*, 2015).

Group III: Received CCl₄ dissolved in olive oil intraperitoneally (Sigma-Aldrich Chemical Co. St. Louis, MO, USA) at a dose of 3 ml/kg twice weekly for 4 weeks (Khan *et al.*).

Group IV: Received CCl₄ intraperitoneally (1ml/kg twice weekly) and rosmarinic acid orally (50 mg/kg/day) for 4 weeks.

Twenty-four hours after last drug regimen, rats were anesthetized with intraperitoneal injection of sodium pentobarbital (35 mg/kg body weight). The chest wall was incised to explore the heart. 5 ml of intracardiac blood was drawn and serum was separated for estimation of serum marker enzymes [aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP)]. The rats were sacrificed by decapitation and liver was removed. Some specimens were perfused with a fixative solution (2 % paraformaldehyde and 2 % glutaraldehyde solution) in 0.1 mol/l phosphate buffer pH7.2 and then sampled for histopathological studies. The level of malondialdehyde (MDA) was then determined in the hepatic tissues. Other specimens were prepared for EM examination.

Assessment of hepatotoxicity. Levels of AST, ALT, and ALP were measured using standard laboratory techniques to assess hepatotoxicity using commercial kits in an Olympus AU400 Chemistry Analyzer (Olympus Corp., Tokyo, Japan). Results are expressed as u/l.

Assessment of oxidative stress. The liver was rinsed with 10 % cold phosphate buffered saline (PBS) solution (PH 7.4) to remove any residual blood clot. Tissues were weighed, homogenized in PBS and centrifuged at 8000 rpm for 15 min at 4 °C to collect supernatant fluids. These supernatant fractions were used to measure the desired markers of oxidative stress.

Analysis of the tissue MDA level as an indicator of lipid peroxidation, was performed by the spectrophotometry (PD-303 Spectrophotometer; APEL CO., LTD., Japan) method. This method was used to obtain a spectrophotometric measurement of the color produced during the reaction to thiobarbituric acid (TBARS Assay Kit, Item No. 10009055, Cayman Chemical Company, Ann Arbor, USA) with MDA at 535 nm according to the method described by Ohkawa *et al.* (1979). The MDA level is expressed as nanomoles per gram of tissue protein.

Antioxidant activity was detected by measuring reduced GSH, GSH-Px, and SOD. The level of reduced GSH (Glutathione Assay Kit, Item No. 703002; Cayman Chemical Company, Ann Arbor, USA) was measured by a kinetic assay using a dithionitrobenzoic acid recycling method that was previously described by Ellman (1959) at 412 nm by spectrophotometer and the results were expressed as micromoles per gram of tissue protein (Ahmida, 2012). The GSH-Px enzyme activity analysis (Glutathione Peroxidase Assay Kit, (ab102530); abcam, Cambridge, United Kingdom) was determined by the procedure that was previously described by Paglia & Valentine (1967) at 340

nm by spectrophotometer and expressed as units per gram of tissue protein. Xanthine/xanthine oxidase assay was used to estimate SOD (Superoxide Dismutase Assay Kit, Item No. 706002; Cayman Chemical Company, Ann Arbor, USA) according to the method of Sun *et al.* (1988) by measuring the amount of reduced nitro blue tetrazolium (NBT) with one unit of SOD, which is defined as the amount of protein that inhibits the rate of NBT reduction by fifty percent. SOD was expressed as units per milligram of tissue protein.

Histological and immunohistochemical examination.

Liver from each animal was kept in 10 % of neutral buffered formalin for 24h. It was then processed, embedded in paraffin wax and sections of 4mm thickness were taken using a microtome. These sections are stained with hematoxylin and eosin (H&E) and are examined under a light microscope, to detect histological changes (Bancroft & Layton, 2012). The histopathological findings in the sections were graded as grade 0, no change; grade 1, mild usually single-cell necrosis in sparse tubules; grade 2, moderate with more than one cell involved in sparse tubules; and grade 3, marked tubules exhibiting total necrosis in almost every power field (Farombi & Ekor, 2006).

Liver sections were immunohistochemically stained to assess immunoexpression of proapoptotic protein (Bax), and antiapoptotic protein (Bcl2). Paraffin sections of liver were cut at 4 um thickness on positively charged slides. Sections were incubated with a monoclonal antibody against Bax and Bcl2 (Dako, Carpinteria California, USA); in a dilution of 1:200. Cells displaying brown precipitation were considered positive for Bax, and Bcl2 expressions.

Transmission electron microscopy examination. The specimens were fixed d in 2.5 % glutaraldehyde for 24 h and subsequently washed in phosphate buffer (pH 7.4), post-fixed in 1 % osmium tetroxide in phosphate buffer (pH 7.4) and dehydrated in increasing concentrations of alcohol. Afterwards, the tissues were washed with propylene oxide and embedded in epoxy-resin embedding media. Semithin sections (1µm thick) were cut with a glass knife on anLKB Nova ultramicrotome, and stained with 1 % toluidine blue and examined by light microscope.

Ultrathin sections (80-90nm) were collected on copper grids, stained with uranyl acetate and lead citrate and finally examined by a JEOL JEM 1400 transmission electron microscope at 120 KV (Bancroft & Layton).

Quantitative morphometric measurement. Leica Qwin 500 C Image analyzer computer system (Leica Imaging System LTD., Cambridge, England) in Central Research Lab, Assiut Faculty of Medicine, Egypt was used to obtain morphometric data in this study. Ten non-overlapping fields in slides of each animal in each group were examined. Measurements were performed on 6µm thick H&E-stained sections to estimate:

1. The diameter of blood sinusoids in control and treated groups at 400× magnification.
2. The diameter of central veins in control and treated groups at 400× magnification.

Statistical Analysis. All analyses were performed using the software Statistical Package for Social Sciences version 17 (SPSS Inc, Chicago, IL, USA). Data was presented as mean±standard deviation (SD). Comparisons between two groups were analyzed by unpaired Student “t” test. Probability of chance (Pvalue)< 0.05 was considered statistically significant.

RESULTS

None of the experimental rats died during the experiment period (4 weeks).

Evaluation of body and liver weights. At the end of the experimental period, the body weight in the CCl4 group was significantly lower than the control group as well as the CCl4+RA group (P<0.05) while no significant difference in body weight was observed between the control, RA and CCl4+RA groups. The liver weight in the CCl4-treated group was significantly higher than the control group as well as the CCl4+RA group (P<0.05). Meanwhile, no significant differences were seen between the control, RA and CCl4+RA groups (Table I).

Table I. Body and liver weights of rats in the different studied groups.

Parameters	Group I (Control)	Group II (RA)	Group III (CCl4)	Group IV (CCl4+RA)
Body weight (g)	225.23±11.48	223.13±11.62	198.51± 12.71 ^a	222.66±10.93 ^b
Liver weight (g)	4.41±0.02	4.38±0.04	6.52± 0.03 ^a	4.15±0.07 ^b

Data is expressed as mean ± standard deviation. Results were statistically analyzed by using Student’s t test at P < 0.05. ap < 0.001 compared with the control group (group I). bp < 0.001 compared with the treated group (group III).

Table II. The level of serum marker enzymes in the normal and experimental groups.

Parameters	Group I (Control)	Group II (RA)	Group III (CCl4)	Group IV (CCl4+RA)
AST (u/l)	46.1 ± 0.23	46.7 ± 0.52	92.3 ± 0.61 ^a	50.2 ± 0.87 ^b
ALT (u/l)	20.3 ± 0.72	19.5 ± 0.17	39.5 ± 0.52 ^a	23.7 ± 0.58 ^b
ALP (u/l)	78.5 ± 0.33	79.4 ± 0.62	112.2 ± 0.31 ^a	82.5 ± 0.44 ^b

Data is expressed as mean ± standard deviation. Results were statistically analyzed by using Student's t test at P < 0.05. ap < 0.001 compared with the control group (group I). bp < 0.001 compared with the treated group (group III).

Biochemical results

Effect of rosmarinic acid on serum marker enzymes.

There was significant increase in serum ALT, AST and ALP in CCl4-treated rats as compared with normal rats. Concomitant administration of rosmarinic acid with CCl4 significantly reduced enzyme levels compared to CCl4-treated group (Table II).

Effect of rosmarinic acid on hepatic oxidative stress.

There was significant increase in MDA level while the level of reduced GSH, GSH-Px, and SOD were noted to be decreased in CCl4-treated rats as compared with normal rats. Concomitant administration of RA with CCl4 significantly prevented CCl4-induced increase in hepatic MDA and reduction of hepatic GSH, GSH-Px, and SOD (P < 0.05) (Table III).

Histological results. The architecture of hepatic tissue of control, and rosmarinic acid-treated rats of H&E-stained sections were more or less similar, showing hepatocytes arranged in cords radiating from the central vein. They are polygonal cells with pale vesicular nuclei and prominent nucleoli. Some hepatocytes were binucleated. They have an eosinophilic granular cytoplasm. Blood sinusoids were found as a network between the plates of hepatocytes converging towards the central vein (Fig. 1A).

In CCl4 group, liver sections revealed dilatation of central veins and blood sinusoids. Many hepatocytes were ballooned with central faint nuclei and vacuolated cytoplasm.

(Fig. 1B). Leucocytic infiltration is seen within the hepatic tissue (Fig. 1C). Some hepatocytes showed early signs of apoptosis, with fragmented nuclei, hazy vacuolated cytoplasm and indistinct cell boundaries. Apoptotic hepatocytes were also detected, showing nuclear and cytoplasmic condensation into deeply stained apoptotic bodies (Fig. 1D). Fibrosis around the central vein and intrahepatic hemorrhage are detected. (Fig. 1E). When rosmarinic acid was administered with CCl4, liver sections appeared somewhat normal in histological architecture. Almost normal hepatocyte appearance was observed both in central as well as in peripheral zones of hepatic lobules (Fig. 1F).

Statistical analysis showed a significant difference in histological score between CCl4 group and control group. However, the group treated with RA showed significant amelioration in the histopathologic score compared with the CCl4 group (Fig. 2).

Immunohistochemical results

Immunostaining of Bax antigen. In control, and rosmarinic acid groups, liver tissues revealed negative immunostaining reaction for Bax. (Fig. 3A). CCl4-treated rats, revealed dark brown granules in their cytoplasm throughout most cells of liver parenchyma (Fig. 3B). Combined CCl4 and rosmarinic acid group showed Bax activity more or less similar to the control group (Fig. 3C).

Table III. Levels of malondialdehyde (MDA), reduced glutathione (GSH), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) in the different studied groups.

Parameters	Group I (Control)	Group II (RA)	Group III (CCl4)	Group IV (CCl4+RA)
MDA (nmol/g tissue protein)	25.7 ± 0.53	24.8 ± 0.09	88.3 ± 0.29 ^a	29.4 ± 0.17 ^b
GSH (µmol/g tissue protein)	3.84 ± 1.47	3.25 ± 1.76	0.14 ± 2.14 ^a	3.55 ± 1.54 ^b
GSH-Px (units/g tissue protein)	27.35 ± 2.12	27.42 ± 2.45	13.95 ± 1.33 ^a	26.91 ± 2.57 ^b
SOD (units/mg tissue protein)	11.54 ± 0.02	11.63 ± 0.02	5.74 ± 0.07 ^a	11.13 ± 0.03 ^b

Data is expressed as mean ± standard deviation. Results were statistically analyzed by using Student's t test at P < 0.05. ap < 0.001 compared with the control group (group I). bp < 0.001 compared with the treated group (group III).

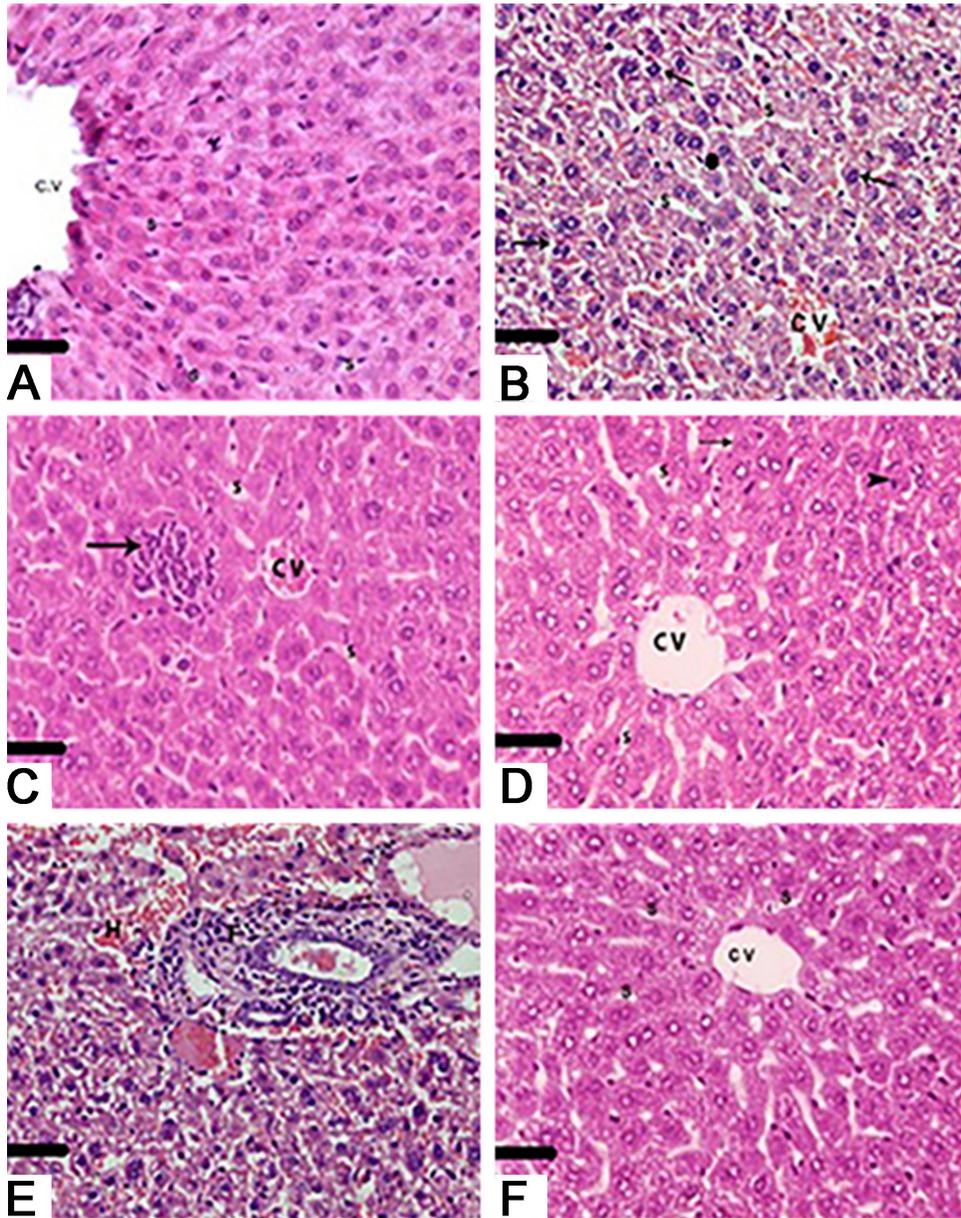


Fig. 1. (Panel A) A photomicrograph of normal architecture of the liver in the control group showing polyhedral shaped hepatocytes with rounded vesicular nuclei and acidophilic cytoplasm radiating from the central vein (CV) and separated by blood sinusoids (s). (H&E, scale bars = 40 μ m). (Panels B-E) Photomicrographs of liver of CCl₄-treated rats. (Panel B) Many of the hepatocytes are ballooned with central faint nuclei and vacuolated cytoplasm (arrows). The central vein (CV) is congested and the blood sinusoids appear dilated (s). (Panel C) Inflammatory cellular infiltration (arrow) is seen within the liver tissue. (Panel D) Some hepatocytes show early signs of apoptosis with fragmented nucleus, hazy vacuolated cytoplasm and indistinct cell boundaries (arrow). Other apoptotic hepatocytes with nuclear and cytoplasmic condensation into deeply stained apoptotic bodies are seen (arrow head) (Panel E) Showing fibrosis around the central vein (F) and intrahepatic hemorrhage (H). (H&E, scale bars = 40 μ m (panels B-E)). (Panel F) A photomicrograph of liver of CCl₄+RA group with normal looking hepatocytes, central vein (CV), and blood sinusoids (s). (H&E, scale bars = 40 μ m).

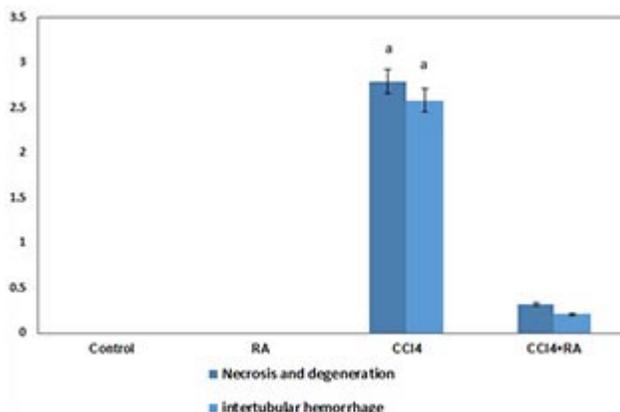


Fig. 2. Mean of histopathological changes in the studied groups tested by using Student's t test. ap < 0.05 vs. control group (group I); bp < 0.05 vs. CCl₄ group (group III). Grade 0, no change; grade 1, mild focal necrosis limited to centrilobular region (Less than 1/4 of affected lobules are necrotic); grade 2, moderate focal and multifocal necrosis found in central to midzonal lobular region (1/2 affected lobules are necrotic); and grade 3, severe hepatocytes necrosis extended from central vein to portal region (whole lobules are necrotic). Total necrosis in almost every power field.

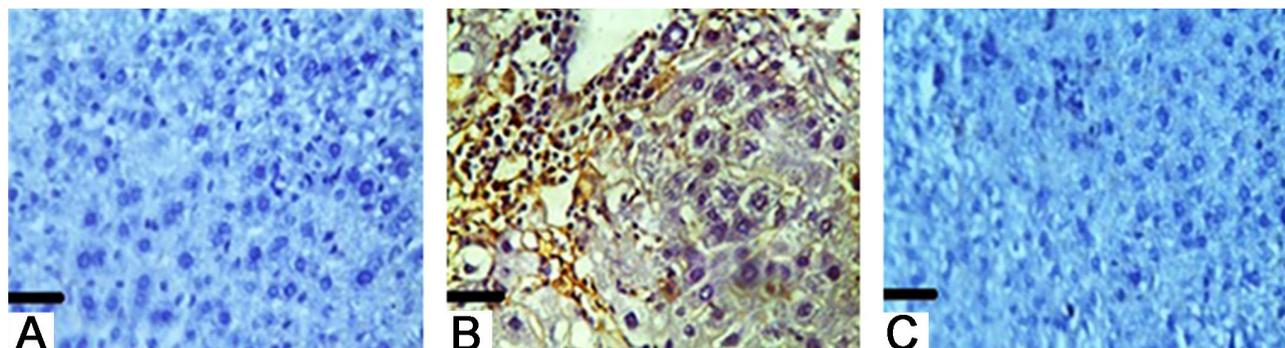


Fig. 3. (A) Negative immunostaining reaction of Bax in the liver tissue of the control group. (B) Strong positive immunostaining reaction of Bax in the liver tissue of the CCl₄-treated group. (C) Negative immunostaining reaction of Bax in the liver tissue of group treated with CCl₄+RA (Bax, immunostaining, scale bars = 40 μm).

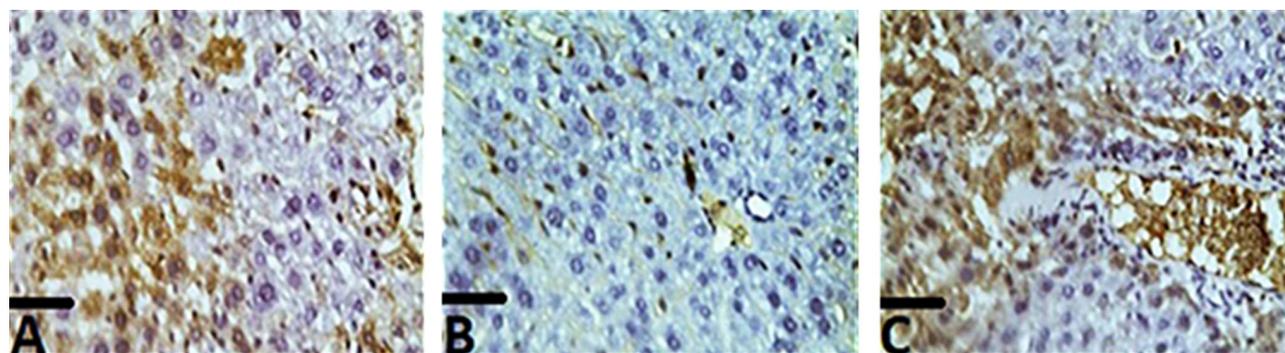


Fig. 4. (A) Strong positive immunostaining reaction of Bcl2 in the liver tissue of the control group. (B) Weak positive reaction of Bcl2 in the liver tissue of CCl₄-treated group. (C) Strong positive immunostaining of Bcl2 in the liver tissue of group treated with CCl₄+RA (Bcl2, immunostaining, scale bars = 40 μm).

Immunostaining of Bcl2. In control, and rosmarinic acid groups, hepatic tissue showed moderate to marked Bcl2 reaction in the cytoplasm of hepatic cells (Fig. 4A). In CCl₄-treated rats, Bcl2 immunostaining was markedly less intense in cytoplasm of hepatic cells (Fig. 4B). Combined CCl₄ and rosmarinic acid group showed increased Bcl2 expression compared with those of CCl₄-treated group (Fig. 4C).

Transmission electron microscopy results

Toluidine blue stain (semithin sections). In control, and rosmarinic acid groups, sections were more or less similar. The hepatocytes were polygonal in shape with pale vesicular nuclei and prominent nucleoli. They have granular cytoplasm. (Fig. 5A). The hepatocytes were arranged in cords radiating from the central vein. The hepatic sinusoids were seen between the hepatocytes (Fig. 5B).

In the CCl₄ group, there were severe histological changes in the form of vacuolated cytoplasm with decreased cytoplasmic granules and pale stained nuclei of hepatocytes (Fig. 5C). Other hepatocytes showed abnormal clumping of

the cytoplasmic granules with shrunken fragmented nuclei and dilated blood sinusoids (Fig. 5D). There were some hepatocytes with early signs of apoptosis. These hepatocytes showed no nuclei with hazy vacuolated cytoplasm and indistinct cell boundaries (Fig. 5E). Mononuclear cellular infiltration appeared around the central vein (Fig. 5F). Some hepatocytes showing markedly vacuolated foamy cytoplasm (Fig. 5G).

When rosmarinic acid was administered with CCl₄, liver sections showed apparently normal hepatic lobular architecture (Fig. 5H).

EM sections (ultrathin sections). In control, and rosmarinic acid groups, examination of ultrathin sections showed the normal hepatic architecture. the hepatocytes had large rounded nuclei that contained abundant euchromatin. The cytoplasm of the hepatocytes was observed to contain abundant cell organelles. The most numerous organelles were mitochondria which appeared rounded or oval in shape, slightly variable in size and distributed throughout the cytoplasm. Parallel cisternae of rough endoplasmic reticulum (RER) were apparent in the perinuclear zone. (Fig. 6A).

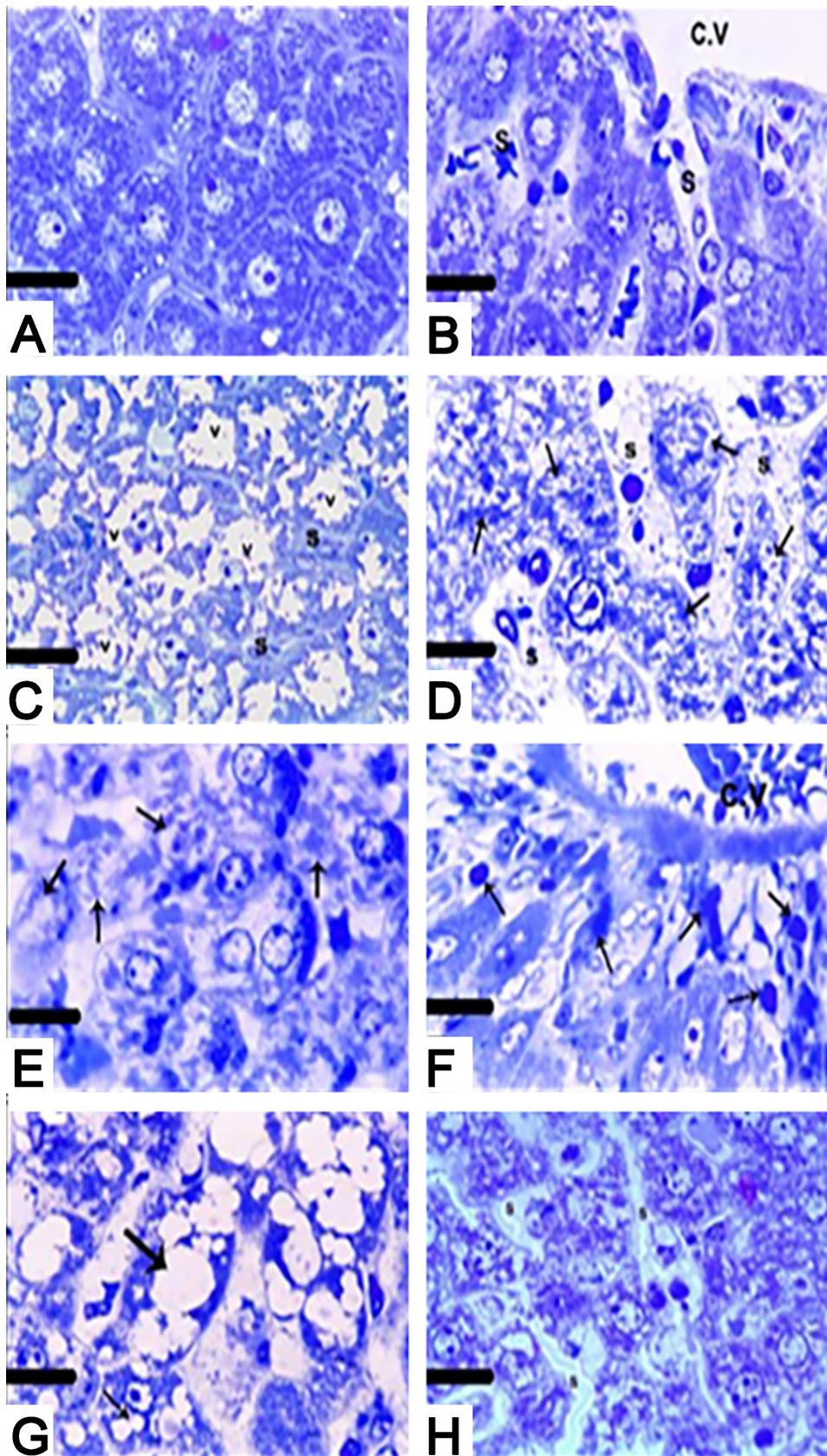


Fig.5. (Panel A-B) Photomicrographs of normal architecture of the liver in the control group.(Panel A) showing hepatocytes with vesicular nuclei, prominent nucleoli and cytoplasmic granules. (Panel B) showing the central vein (C.V) with cords of hepatocytes radiating from it. Blood sinusoids (S) are seen between the hepatocytes. (Toulidine blue, scale bars = 100 μ m). (Panels C-G) Photomicrographs of liver of CC14-treated rats. (Panel C) showing hepatocytes with markedly vacuolated cytoplasm (V), depleted cytoplasmic granules and pale stained nuclei. (Panel D) showing some hepatocytes with fragmented nuclei and abnormal clumping of the cytoplasmic granules (arrows). Dilated blood sinusoids are seen (S). (Panel E) showing some hepatocytes with early signs of apoptosis (arrows). These hepatocytes showed fragmented nuclei with hazy vacuolated cytoplasm and indistinct cell boundaries. (Panel F) showing mononuclear cellular infiltration (arrows) around central vein. (Panel G) showing markedly vacuolated foamy cytoplasm of hepatocytes [microvacuoles (thin arrow) & macrovacuoles (thick arrow)]. (Toulidine blue, scale bars = 100 μ m (panel C-G)). (Panel H) A photomicrograph of liver of CC14 + RA group with normal looking hepatocytes, and blood sinusoids. (Toulidine blue, scale bars = 100 μ m).

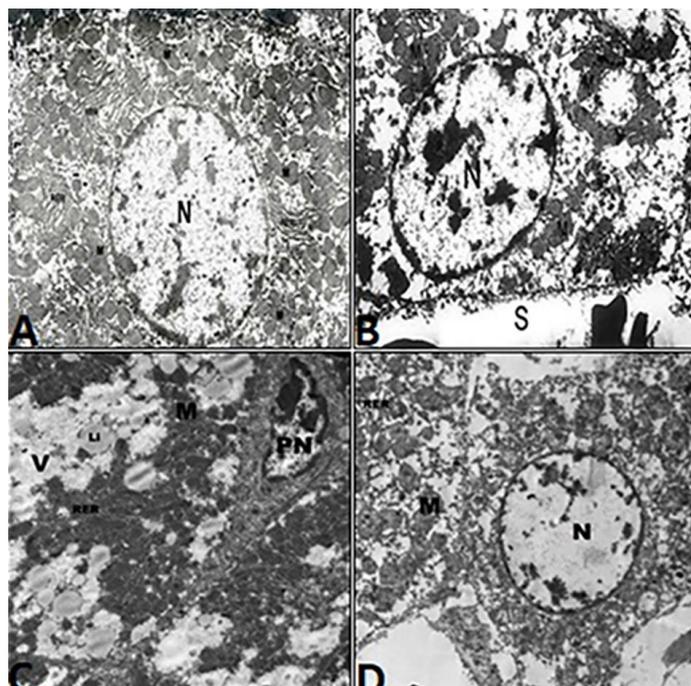


Fig. 6. (Panel A) An electron photomicrograph of normal architecture of the liver in the control group showing a hepatocyte with a large rounded nucleus (N) with abundant euchromatin, and mitochondria (M) that appear slightly variable in shape and size. Parallel cisternae of rough endoplasmic reticulum (RER) are apparent in the perinuclear zone. (EM, X 4000). (Panel B-C) Electron photomicrographs of liver of CCl4-treated rats. (Panel B) showing a hepatocyte with highly vacuolated rarefied cytoplasm. The nucleus (N) has clumped chromatin with abnormal irregular nuclear envelope. Swollen mitochondria (M) and dilated rough endoplasmic reticulum (RER) appear in the perinuclear zone. (Panel C) showing pyknotic nucleus (PN) and highly vacuolated cytoplasm (V). Some lipid droplets (Li) are seen in the cytoplasm. (EM, X 4000 (panel B-C)). (Panel D) An electron photomicrograph of liver of CCl4 + RA group showing normal rounded euchromatic nucleus (N), parallel flattened cisternae of rough endoplasmic reticulum (RER), and normal mitochondria (M). (EM, X 4000).

In the CCl4 group, examination of ultrathin sections of liver showed alteration in the normal structure. The hepatocyte showed highly vacuolated rarefied cytoplasm. The nucleus (N) had clumped chromatin with abnormal irregular nuclear envelope. Swollen mitochondria (M) and dilated rough endoplasmic reticulum (RER) appeared in the perinuclear zone (Fig. 6B).

Another hepatocyte showed pyknotic nucleus (PN) and highly vacuolated cytoplasm (V). Some lipid droplets (Li) are seen in the cytoplasm (Fig. 6C). When rosmarinic acid was administered with CCl4, ultrathin sections of liver showed ultrastructure more or less similar to the control group (Fig. 6D).

Quantitative morphometric measurement. Diameters of blood sinusoids and central veins were significantly increased in CCl4-treated rats. Combined CCl4 and RA group showed significantly decreased diameter of blood sinusoids and central veins compared to CCl4-treated group. Rats treated with RA alone did not show difference when compared to normal values (Table IV).

DISCUSSION

It is well known that CCl4 is an experimental hepatotoxin that induces characteristic centrilobular degenerative lesions and lastly causes liver fibrosis. The liver damage induced by CCl4 is similar to that due to hepatitis. Thus, evaluation of the prevention of CCl4-induced liver damage has widely been used as an indicator of the liver protective ability of different drugs (Abdel-Razik *et al.*, 2006).

Several natural agents have been used to ameliorate drugs toxicity. In this research, we were interested in studying the protective effect of rosmarinic acid (RA), a phenolic compound, against CCl4-induced hepatotoxicity as they are well known as highly efficient scavengers of reactive oxygen species (Bakirel *et al.*, 2008).

The administration of CCl4 in the current study caused a significant decrease in body weight and a significant increase in liver weight. Dutta *et al.* (2018) reported that the body weight was decreased while the liver weight was increased in CCl4-treated mice. However, in combined CCl4 and RA group, Body and liver weights were reversed to the control levels.

Table IV. The diameter of blood sinusoids (BS) and central veins (CV) in the different studied groups.

Parameters	Group I (Control)	Group II (RA)	Group III (CCl4)	Group IV (CCl4+RA)
Diameter of BS (um)	8.25±0.18	8.51±0.29	19.63±0.44 ^a	8.82±0.74 ^b
Diameter of CV (u m)	95.42±2.67	95.65±2.14	214±1.87 ^a	96.11±2.59 ^b

Data is expressed as mean ± standard deviation. Results were statistically analyzed by using Student's t test at P < 0.05. ap < 0.001 compared with the control group (group I). bp < 0.001 compared with the treated group (group III).

Marked increase in the level of serum liver enzymes (AST, ALT, and ALP) was found in CCl₄-treated rats. The elevated levels of these biochemical parameters are direct reflection of changes in the structural integrity of the liver. CCl₄ causes acute hepatocyte injuries, alters membrane integrity and as a result, enzymes in hepatocytes leak out. These results are in agreement with Eman & Naglaa (2016) who showed that injecting the animals with CCl₄ resulted in severe liver damage evidenced by sharp increase in hepatic lipid peroxidation and both ALT and AST. Another study evidenced a marked increase in the levels of serum liver enzymes (AST, ALT and ALP) in rats treated with CCl₄ alone (Adewale *et al.*, 2014).

Levels of serum liver enzymes were significantly decreased in combined CCl₄ and rosmarinic acid group compared to the CCl₄ group. These results are in agreement with Mahgoub (2016) who declared that RA reduced the level of liver marker enzymes in rats treated with ethanol. Another study reported that RA (50 and 100 mg/kg) treatment protected against liver damage induced by acetaminophen as indicated by the improvement in the biochemical parameters (Hasanein & Sharifi, 2017). Lucarini *et al.* (2014) found that RA prevented acetaminophen-induced elevation of ALT, AST, and ALP levels in a dose-dependent manner.

Significant elevation in the level of malondialdehyde (MDA) was detected in CCl₄-treated rats as compared with normal rats. While, the levels of reduced GSH, GSH-Px, and SOD were significantly decreased. MDA is a marker for oxidative stress. It results from lipid peroxidation of polyunsaturated fatty acids. ROS degrade polyunsaturated lipids, forming MDA. Our findings were in agreement with Eman & Naglaa who found that administration of CCl₄ in mice markedly depleted the antioxidant enzymes (CAT, GSH-Px and SOD) and significantly increased the lipid peroxidation that is expressed by high TBARS content. Another study reported that CCl₄ treatment in rats significantly decreased the activity of SOD, GSH-Px, GSH while increased TBARS content in liver samples

The level of MDA was significantly decreased and the levels of SOD, GSH, and GSH-Px were significantly increased in the combined CCl₄ and RA group compared to the CCl₄ group. Mahgoub found that the treatment of ethanol-intoxicated animals with RA significantly ameliorated CAT, GPx and GSH content. Hasanein & Sharifi indicated that RA (50 and 100 mg/kg) reduced the formation of MDA in the liver and protected the total tissue antioxidant capacity.

Histological examination of H&E-stained sections of CCl₄-treated rats revealed prominent histological alterations

in the liver. These changes were in the form of cytoplasmic vacuolation in the hepatocytes occupying the centrilobular zone, dilatation of the central veins and blood sinusoids and localized foci of necrosed hepatocytes surrounded by inflammatory cellular infiltration. These results are supported by Eman & Naglaa who revealed severe structural loss of hepatic tissue distinctive by dense periportal and lobular lymphocytic infiltrate with diffused pyknotic nuclei within necrotic hepatocytes in animals treated with CCl₄. Another study found that histopathological examination of rats treated with CCl₄ alone showed massive tissue necrosis, congested central vein, fatty degeneration and infiltration by inflammatory cells (Adewale *et al.*).

The histopathological findings obtained from the combined CCl₄ and rosmarinic acid group showed marked amelioration compared to CCl₄ group as most of the hepatocytes, central veins, blood sinusoids and portal triads appeared more or less similar to the control group. The current results are in agreement with Domitrovic *et al.* (2013) who reported that rosmarinic acid exerted hepatoprotective effects against CCl₄-induced liver damage.

Ultrastructural changes were found in CCl₄ group in the form of dilated rough endoplasmic reticulum, mitochondrial swelling, irregularity of the outer nuclear envelope with clumping chromatin, and vacuolization and rarefaction of the cytoplasm. Hassanein *et al.* (2016) found that ultrastructural changes as a result of CCl₄ treatment were in the form of dilatation of endoplasmic reticula, increased extracellular matrix, and formation of numerous perinuclear fatty globules. Another study revealed that induction of liver toxicity by CCl₄ resulted in mitochondrial swelling with fragmentation and destruction of their crista, vesiculation in the cytoplasm of hepatocytes, dilatation of the endoplasmic reticulum, and expansion of perinuclear space with a pyknotic changed nucleus and margined chromatin (Hovnanyan *et al.*, 2014). Tasci *et al.* (2008) showed that CCl₄ administration in rats caused mitochondrial edema, dilatations with focal breaks in rERs, extensive margination and clumping chromatin in nuclei, and large vacuolization of hepatocyte cytoplasm. The ultrastructure of hepatocytes obtained from the combined CCl₄ and rosmarinic acid group was closer to the picture of normal cells.

Morphometric studies in the present study revealed significant increase in the diameter of central veins and blood sinusoids in CCl₄-treated group. This may be attributed to the increase in the level of nitric oxide (NO). Nitric oxide-induced vasodilatation was also reported by Hingorani (2003). In the group treated with combined CCl₄ and RA, Morphometric studies revealed normal diameter of central

veins and blood sinusoids. This can be attributed to the antioxidant properties of RA.

It could be concluded that Rosmarinic acid has hepatoprotective effects against CCl₄-induced hepatotoxicity as indicated by biochemical, histological, immuno histochemical and ultrastructural results.

BADAWI, M. S. Estudio sobre la actividad antioxidante del ácido rosmarínico contra la toxicidad hepática inducida por tetracloruro de carbono en ratas albinas macho adultas. *Int. J. Morphol.*, 40(1):157-167, 2022.

RESUMEN: El tetracloruro de carbono (CCl₄) es un producto químico fabricado y no se encuentra de forma natural en el medio ambiente. CCl₄ es un líquido transparente que se evapora fácilmente; tiene un olor dulce. CCl₄ es tóxico para el hígado de los mamíferos y es hepatocarcinogénico tanto en ratas como en ratones. El romero (*Rosmarinus officinalis*) se usa comúnmente como condimento y agente aromatizante en el procesamiento de alimentos. Es conocido por sus propiedades antioxidantes. El presente estudio tuvo como objetivo investigar la actividad antioxidante del ácido rosmarínico (RA) sobre la toxicidad hepática inducida por CCl₄ en ratas albinas macho adultas. Se dividieron cuarenta ratas albinas macho adultas en 4 grupos con 10 ratas en cada grupo. Grupo I (grupo control). Los animales del grupo II recibieron AR a una dosis de 50 mg / kg / día por sonda oral durante 4 semanas. Los animales del grupo III recibieron CCl₄ por vía intraperitoneal a una dosis de 3 ml / kg dos veces por semana durante 4 semanas. Los animales del grupo IV recibieron CCl₄ Plus RA. Al final del experimento, las muestras de hígado se procesaron para estudios histológicos, inmunohistoquímicos, EM y bioquímicos. La administración de AR eliminó las enzimas hepáticas séricas elevadas (AST, ALT y ALP), el nivel elevado de MDA y la inmunexpresión de la proteína proapoptótica (Bax) inducida por CCl₄. Aumentó el glutatión reducido (GSH), glutatión peroxidasa (GSH-Px), la superóxido dismutasa (SOD) y la inmunexpresión de la proteína antiapoptótica (Bcl2). También mejoró los cambios histológicos y ultraestructurales inducidos por CCl₄. El ácido rosmarínico puede tener efectos protectores contra la hepatotoxicidad inducida por CCl₄, tal como lo indican los resultados bioquímicos, histológicos, inmunohistoquímicos y ultraestructurales.

PALABRAS CLAVE: Tetracloruro de carbono; Toxicidad hepática; Antioxidantes; Ácido rosmarínico; Estrés oxidativo; EM.

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