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# *In vivo* protective effect of *Rosmarinus officinalis* oil against carbon tetrachloride ($CCl_4$)-induced hepatotoxicity in rats

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ABSTRACT

Exposure to environmental pollutants such as carbon tetrachloride ($CCl_4$) causes liver injuries. There are claims that extracts from *Rosmarinus officinalis* protect from such injuries. This study aimed to explore the hepatoprotective effects of cold-pressed *R. officinalis* oil (CPRO) against $CCl_4$-induced liver toxicity in the experimental rats. Fatty acids and bioactive lipids of CPRO were analyzed. CPRO was orally administered to rats in two doses (100 and 200 mg/kg) along with $CCl_4$ (1 mL/kg in olive oil) for 8 weeks. Indices of liver and kidney functions, lipid profile and oxidation were evaluated in rats' serum and tissues. In CPRO the percentages of polyunsaturated, monounsaturated, and saturated fatty acids were 42.3%, 41.7%, and 15.8%, respectively. CPRO contained high amounts of total phenolic compounds (7.20 mg GAE/g). α-, β-, γ- and δ-tocotrienols accounted for 18, 12, 29, and 158 mg/100 g CPRO, respectively, while α-, β-, γ- and δ-tocopherols accounted for 291, 22, 1145, and 41 mg/100 g CPRO, respectively. The $LD_{50}$ at 24 h was 5780 mg/kg. Treatment with 200 mg/kg CPRO caused a decrease in creatinine, urea and uric acid levels to 0.66, 28.3 and 3.42 mg/dL, respectively. After 8 weeks of administration, levels of total lipids (TL), total cholesterol (TC), total triacylglycerol (TAG), low dentistry lipoprotein-cholesterol (LDL-C) and very low dentistry lipoprotein-cholesterol (VLDL-C) were decreased to 565, 165, 192, 75.6 and 38.5 mg/L, respectively. Malondialdehyde levels in liver were reduced and glutathione levels were elevated in CPRO-treated rats. CPRO reduced the activity of ALT, AST, and ALP as well as kidney function markers, lipid and protein profiles. Histopathological examination of liver indicated that CPRO administration reduced fatty degenerations, cytoplasmic vacuolization and necrosis. CPRO possessed a hepatoprotective effect against $CCl_4$-induced toxicity, mediated possibly due to the antioxidant traits of CPRO. CPRO contains high levels of phenolics and tocols, which is a scavenger of reactive species making the oil a promising material for functional foods and pharmaceuticals.

## 1. Introduction

The liver plays a great role in the metabolism of *endo-* and *exo-*genous compounds. xposure to carcinogens could overpower the antioxidant defense system and cause liver damage [1,2]. Liver could be a subject to several substances resulting in potential injuries phalloidin, galactosamine, ethanol, and carbon tetrachloride $CCl_4$ [3,4]. As $CCl_4$ is a chemical lead to hepatotoxicity, lab animals are commonly administered with $CCl_4$ to attain induction of toxic liver injury [5,6]. The damage to cellular molecules including lipids, enzymes and DNA occurs when free radicals produced in the body lead to oxidative stress status [7–9]. After a hepatic injury, the damaged hepatocytes, metabolites of toxicants, and infiltrating inflammatory cells are strong activators of Kupffer cells that release agents including cytokines as well as reactive oxygen species (ROS) such as malondialdehyde (MDA) and nitric oxide [6,10,11]. Those highly reactive oxygen intermediates induced in the body are proved pathogenic in liver injury [12]. In addition, biochemical markers such as alkaline phosphatase, alanine transaminase, bilirubin, aspartate transaminase, total cholesterol and total triglycerides were increased in serum [4]. ROS have an important role in liver diseases, while antioxidants could prevent hepatic damage through antiradical potential and increase the efficiency of antioxidant enzymes such as catalase, superoxide dismutase, and phospholipid hydroperoxide glutathione peroxidase [12–14]. Antiradical potential against free radicals by defense systems might reduce the fibrosis in the liver tissues [15,16]. The role of antioxidant enzymes *in vivo* was studied deeply

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[17,18].

Oxidative stress stands as a main topic of interest as it is linked with different diseases such as cancer and liver dysfunction [4,19,20]. Plant secondary metabolites are crucial for defense activities (i.e. antioxidant traits), therefore evaluating the role of natural bioactive compounds in combatting diseases was increased. The growing interest towards phytochemicals could be due to the increasing doubtful approach towards reported carcinogenic traits of synthetic antioxidants [4,6]. Phenolics-rich plants achieved great attention to nutritionists and food manufacturers as they have antioxidant traits by preventing free radical's generation and preventing oxidative-stress related diseases [21–26]. Traditional drugs utilized to treat liver diseases being inadequate and could cause serious adverse impacts. Plants have defense systems such as antioxidant enzymes and phenolic compounds that protect the plant itself and others from damage [4]. Interest in the role of phytochemicals and naturally-originated constituents in the treatment of liver damage was significantly increased. Several bioactive phytochemicals were reported to have a protective impact against liver damage [6,25,27]. Phytochemicals and natural antioxidants protected the liver against lipid oxidation and impairment in antioxidant status caused by $CCl_4$ [3,6,28]. Natural bioactive compounds from plant extracts were effective in preventing oxidative stress-related liver pathology due to synergisms and interactions [29,30].

Extracts, bioactive compounds, and oils from oilseeds, fruits, vegetable and medical plants showed high antioxidant traits that could act against hepatic damage. *Rosmarinus officinalis* L. (Lamiaceae) cold-pressed oil, which selected in the present investigation, is one such candidate. *R. officinalis* is a native Mediterranean green shrub with pale blue flowers that bloom in early spring [2,31]. *R. officinalis* extracts and essential oil has been known to have therapeutic potential in the treatment of diseases such as peptic ulcers [32], inflammation [33] and cancer [2]. Rosemary extracts exhibited hepatoprotective impacts against hepatotoxic agents including t-BHP [34], $CCl_4$ [1], and cyclophosphamide [1]. In addition, *R. officinalis* exhibited a protective effect against Azathioprine-induced liver injury in rats and blocked serum elevated levels of aspartate aminotransferase and alanine aminotransferase [35]. Extract from *R. officinalis* leaves mitigated cyclophosphamide-induced [2] and creosote-induced [36] hepatotoxicity in rats.

*R. officinalis* contains antioxidants such as rosmarinic acid, diterpenoids like carnosic acid, carnosol, rosmanol, and epirosmanol [37] as well as tocols and carotenoids [35,38]. The antioxidant activities of *R. officinalis* extract have been associated with phenolic diterpenes that scavenge singlet oxygen, hydroxyl radicals and lipid peroxyl radicals [39]. Interest in environmental-friendly techniques resulted in a big market of natural products [40]. Cold-pressing is a simple environmentally safe technique, that requires no chemical or thermal treatment [31,41,42]. Cold-pressed oils are commercially sold even though the functional properties and composition of only a few of them were investigated [25,41].

The protective impact of cold-pressed rosemary oil (CPRO) on $CCl_4$-induced oxidative damage in rats was not reported yet. In this work, the protective impact of CPRO against $CCl_4$-induced hepatotoxicity and oxidative stress in rats was studied. The extent of $CCl_4$-induced liver damage was monitored by screening different biochemical and histopathological parameters.

## 2. Material and methods

### 2.1. CPRO and materials

CPRO was acquired from a local market (Zagazig city, Egypt). CPRO has specifications and codex standard approved by the Egyptian Organization for Standardization and Quality (approval number 66/92). $CCl_4$ and tocols standards were acquired from Merck (Darmstadt, Germany). Kits and chemicals were of the analytical grade and acquired from Sigma (St. Louis, MO).

### 2.2. Methods

#### 2.2.1. Analysis of fatty acids, tocols and phenolic compounds in CPRO

According to Arens et al. [43], fatty acids of CPRO were transesterified into fatty acids methyl esters (FAME) using *N*-trimethylsulfoniumhydroxide (Macherey-Nagel, Germany). FAME was analyzed on a Shimadzu GLC-14A equipped with FID and C-R4AX chromatopac integrator (Kyoto, Japan). The flow rate of the carrier gas (helium) was 0.6 mL/min, and split with a ratio of 1:40. A sample of 1 μL was injected into a Supelco SPTM-2380 capillary column (Bellefonte, PA, USA). Injector and FID temperatures were set at 250 °C. The initial column temperature was 100 °C, programmed to rise by 5 °C/min up to 175 °C and kept for 10 min at 175 °C, then programmed to rise by 8 °C/min up to 220 °C and kept for 10 min at 220 °C.

For tocols analysis, a solution of 250 mg of CPRO in 25 mL *n*-heptane was used for HPLC [41,44]. HPLC analysis was conducted using a Merck Hitachi low-pressure gradient system, fitted with an L-6000 pump, a Merck-Hitachi F-1000 Fluorescence spectrophotometer (detector wavelength was set at 295 nm for excitation, and at 330 nm for emission) and a D-2500 integration system; 20 μL of the samples were injected onto HPLC column 25 cm x4.6 mm ID (Merck, Germany) with 1.3 mL/min flow rate. Mobile phase was *n*-heptane/*tert*-butyl methyl ether (99:1, v/v).

For phenolics analysis, CPRO (1 g) was dissolved in *n*-hexane (5 mL) and mixed with 10 mL methanol: water (80:20, v/v) for 2 min in a vortex [45]. After centrifugation at 3000 rpm for 10 min, the hydroalcoholic extracts were separated from the lipid phase using a Pasteur pipette then concentrated *in vacuo* at 30 °C. Oil residue was redissolved in 10 mL methanol: water (80:20, v/v) and extraction were repeated twice. Hydroalcoholic extracts were redissolved in acetonitrile (15 mL) and the mixture was washed three times with *n*-hexane (15 mL each). Purified phenolics in acetonitrile were concentrated *in vacuo* at 30 °C and dissolved in methanol for further analysis. Aliquots of phenolic extracts were evaporated under nitrogen. The residue was dissolved in 0.2 mL water and 1 mL of diluted (1:30, v/v) Folin-Ciocalteu's reagent. After 3 min, 0.8 mL of 7.5% sodium carbonate was added. After 30 min, the absorbance was measured at 765 nm using a UV-260 spectrophotometer (Shimadzu, Japan). Gallic acid was used for the calibration, and the results of triplicate analyses were expressed as ppm of gallic acid.

#### 2.2.2. Experimental animals

Experimental procedures involving animals and their care were performed in conformity with the institutional guidelines and the Guidelines for Care and Use of Laboratory Animals in Biomedical Research as adopted and promulgated by the World Health Organization. Permission was obtained from Ethical Committee (Faculty of Agriculture, Banha University, protocol number 2016-2) Healthy adult male albino rats (Wister Strain) of the approximately same age, weighing approximately 120–140 g, were purchased from Organization of Biological Products and Vaccines (Helwan Farm, Egypt). Animals were housed under ambient temperature (25 ± 2 °C) with 50 ± 5% relative humidity and a 12 h light-dark cycle. Rats received standard pellet diet comprised of 20% protein, 5% fat, 4.5% fiber, 8% ash, 2% calcium, 0.6% phosphorus, 3.4% glucose, 2% vitamins and water *ad libitum*.

#### 2.2.3. Experimental design

Twenty-four rats were used to study the protective role of CPRO on the $CCl_4$-induced hepatotoxicity in rats [26]. $CCl_4$ was mixed with olive oil in the ratio of 1:1 (w/w) and used for hepatoprotective potential against $CCl_4$-induced liver damage. Rats were divided randomly into 4 groups (6 rats each) and treated as follows:

**Group 1** (negative control group): animals fed on a standard synthetic diet for 8 weeks.

**Group 2** (positive control group): animals fed on a standard diet

and received with an equal mixture of $CCl_4$ and olive oil orally (three times a week) by gastric gavages at a dose of 1 mL/kg (bw) for 8 weeks.

**Group 3**: animals fed on a standard diet and received CPRO orally (three times a week) by gastric gavage at a dose of 100 mg/kg, simultaneously with an equal mixture of $CCl_4$ and olive oil by gastric gavage at a dose of 1 mL/kg during the last 4 weeks.

**Group 4**: animals fed on a standard diet and received CPRO orally (three times a week) by gastric gavage at a dose of 200 mg/kg, simultaneously with an equal mixture of $CCl_4$ and olive oil by gastric gavage at a dose of 1 mL/kg during the last 4 weeks.

#### 2.2.4. Blood sampling and biochemical analysis

Blood samples were collected at the end of the experiment and obtained from the retro-orbital plexus veins from the individual rat by means of fine capillary heparinized tubes. Samples were collected and allowed to clot, serum was separated by centrifugation at 3000 rpm for 15 min. Serum was used to investigate the biochemical parameters including liver and kidney function tests and serum lipid profile.

Activities of liver enzymes including aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), serum total bilirubin (TB), direct bilirubin (DB), total protein and serum albumin were determined according to Reitman and Frankel [46]; Tietz et al. [47]; Doumas et al. [48], and Doumas et al. [49], respectively. Globulin was calculated by subtracting the albumin from serum total protein. Kidney function parameters including urea, uric acid and creatinine were measured according to Amer et al. [50]. Lipid profile including total lipids (TL), triglycerides (TAG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), and very low-density lipoprotein cholesterol (VLDL-c) was determined according to Ramadan et al. [51].

#### 2.2.5. Antioxidant markers

Liver samples were washed with ice-cold saline to remove excess blood. Liver tissue was homogenized in cold 0.1 M potassium phosphate saline (pH = 7.4) at a concentration of 10% (w/v). The homogenate was centrifuged at 5000 rpm for 10 min at 4 °C to obtain the supernatant, which was used to investigate antioxidant markers. Lipid peroxides (malondialdehyde, MDA) were measured according to Uchiyama and Mihara [52]. Reduced glutathione (GSH) content in the liver was measured spectrophotometrically using Ellman's reagent (DTNB) following Moron et al. [53]. The absorbance was read at 412 nm and the standard graph was drawn using different concentrations of a standard GSH solution and GSH contents were calculated as nM per mg of tissue protein.

#### 2.2.6. Histopathological examination

Small tissue specimens were collected from the fresh liver tissue of rats and fixed in 10% neutral buffered formalin [25]. After fixation, thin paraffin sections were routinely prepared and stained with Hematoxylin and Eosin stain (H&E) for the histopathological lesions in hepatic and renal tissues. Liver sections were graded numerically to assess the degree of histopathological features of acute hepatic injury.

#### 2.2.7. Statistical analysis

Data were analyzed by one-way ANOVA. Duncan's new multiple-range test was used to resolve the difference between treatment means. All statistical analyses were performed using the statistical software SPSS 11.0 (SPSS Ltd., Surrey, UK). $P < 0.05$ was considered statistically significant. Ratio values were not arcsin transformed before statistical analysis

## 3. Results

### 3.1. Fatty acids, tocols and phenolic compounds in CPRO

Nine fatty acids were identified in CPRO. The oil contained high amounts of linoleic acid (41.7%), and oleic acid (41.2%). Palmitic acid (8.9%) and stearic acid (5.96%) were the main saturated fatty acids. The levels of polyunsaturated (PUFA), monounsaturated (MUFA), and saturated fatty acids (SFA) were 42.3%, 41.7%, and 15.8%, respectively. CPRO contained significant levels of MUFA and PUFA wherein U/S ratio was 5.30. The fatty acid profile makes RO especially useful in nutrition. CPRO contained high levels of unsaponifiable (25.10 g/kg). The amounts of α-, β-, γ- and δ-tocotrienols were 18, 12, 29, and 158 mg/100 g oil, respectively, while α-, β-, γ- and δ-tocopherols in CPRO were 291, 22, 1145, and 41 mg/100 g oil, respectively. The main tocol homolog was γ-tocopherol, that makes up more than 66% of tocols content. CPRO contained also high levels of total phenolic compounds (7.20 mg GAE/g). The amounts of tocols and phenolics determined in CPRO suggests that CPRO may effectively resist lipid peroxidation.

### 3.2. Influence of CPRO on liver enzymes, kidney functions, protein profile, lipids profile and antioxidant markers in CCl4-induced liver injury in rats

$LD_{50}$ of CPRO was 5780 mg/kg based on a 24 h oral toxicity investigation. In a long-term toxicity investigation using a different concentration of CPRO (100, 200, and 400 mg/kg, p. o.), only 400 mg/kg induced a decrease in the body weight. The impact of two doses of CPRO on liver enzymes in $CCl_4$-treated animals was studied and the data are given in Table 1. The defensive aptitude of CPRO was validated by measuring the serum levels of AST, ALT, and ALP in administered rats, whose levels rise under oxidative stress. The high concentrations of serum markers were reduced when administered with CPRO low dose (100 mg/kg) in comparison to control. A more significant reduction occurred when treated with high dose of CPRO (200 mg/kg). After $CCl_4$ injection, ALT, AST, ALP enzymes activities in the $CCl_4$-treated group (groups 2) were significantly increased ($p < 0.05$), as compared to negative control (groups 1). Administration of CPRO attenuated the increased levels of ALT, AST, and ALP enzymes induced by $CCl_4$ and caused a subsequent recovery toward normalization comparable to the control (group1). Treatment of animals with both doses of CPRO reduced the activities of serum ALT, AST, and ALP enzymes as compared to the positive group. Moreover, levels of DB and TB were also reduced upon CPRO treatments (groups 3 and 4).

Table 2 shows the effect of CPRO on the protein profiles (total protein, albumin (A), globulin (G) and A/G ratio) of $CCl_4$-treated rats. Results revealed in general that $CCl_4$ treatment (group 2) reduced the protein parameters; while treatment with both doses of CPRO increased protein profiles to levels resemble negative control (group 1). The level

**Table 1**
Changes in liver enzymes in $CCl_4$-induced oxidative damage in rat liver as affected by CPRO.

| Group | Treatment | ALT (U/mL) | AST (U/mL) | ALP (U/L) | TB (mg/dL) | DB (mg/dL) |
|---|---|---|---|---|---|---|
| 1 | Negative control (normal) | 28.33 ± 2.76$^{c}$ | 29.00 ± 8.23$^{b}$ | 95.11 ± 3.29$^{bc}$ | 0.751 ± 0.07 $^{b}$ | 0.123 ± 0.02 $^{c}$ |
| 2 | Positive control ($CCl_4$) | 138.33 ± 2.76$^{a}$ | 171.66 ± 8.23$^{a}$ | 293.33 ± 3.29$^{a}$ | 1.65 ± 0.07$^{a}$ | 0.623 ± 0.02 $^{a}$ |
| 3 | CPRO (100 mg/kg) + $CCl_4$ | 38.33 ± 2.76$^{b}$ | 33.33 ± 8.23$^{b}$ | 100.23 ± 3.29$^{b}$ | 0.91 ± 0.07$^{b}$ | 0.22 ± 0.02 $^{b}$ |
| 4 | CPRO (200 mg/kg) + $CCl_4$ | 36.67 ± 2.76$^{bc}$ | 25.67 ± 8.23$^{b}$ | 85.30 ± 3.29$^{c}$ | 0.97 ± 0.07 $^{b}$ | 0.17 ± 0.02$^{bc}$ |

$^{a, b, c, \cdots}$ *etc.* There is no significant difference ($P > 0.05$) between any two means with the same letter in each column.

**Table 2**
Effect of CPRO on kidney function indicators and protein profile in $CCl_4$-induced injury in rat.

| Group | Treatment | Creatinine (mg/dL) | Urea (mg/dL) | Uric acid (mg/dL) | T-protein (g/dL) | Albumin (g/dL) | Globulin (g/dL) | A/G ratio |
|---|---|---|---|---|---|---|---|---|
| 1 | Negative control (normal) | 0.716 ± 0.026 $^{b}$ | 25.3 ± 1.11 $^{b}$ | 3.63 ± 0.18 $^{b}$ | 6.50 ± 0.203 $^{a}$ | 3.78 ± 0.016$^{b}$ | 2.72 ± 0.192$^{a}$ | 1.40 ± 0.109$^{a}$ |
| 2 | Positive control ($CCl_4$) | 1.85 ± 0.026 $^{a}$ | 56.3 ± 1.11$^{a}$ | 7.93 ± 0.18 $^{a}$ | 5.40 ± 0.203$^{b}$ | 3.01 ± 0.016$^{c}$ | 2.38 ± 0.192$^{a}$ | 1.31 ± 0.109$^{a}$ |
| 3 | CPRO (100 mg/kg) + $CCl_4$ | 0.69 ± 0.026 $^{b}$ | 28.3 ± 1.11$^{b}$ | 3.30 ± 0.18$^{b}$ | 6.60 ± 0.203 $^{a}$ | 3.78 ± 0.016$^{b}$ | 2.81 ± 0.192$^{a}$ | 1.34 ± 0.109$^{a}$ |
| 4 | CPRO (200 mg/kg) + $CCl_4$ | 0.66 ± 0.026 $^{b}$ | 28.3 ± 1.11$^{b}$ | 3.42 ± 0.18$^{b}$ | 6.66 ± 0.203 $^{a}$ | 3.91 ± 0.016$^{a}$ | 2.74 ± 0.192$^{a}$ | 1.43 ± 0.109$^{a}$ |

$^{a, b, c, \cdots}$ *etc*There is no significant difference ($P > 0.05$) between any two means with the same letter in each column.

of A and G was measured in the serum to evaluate the protective impact of CPRO on liver metabolism (Table 2). It was found that albumin and globulin levels in serum decreased with $CCl_4$ administration. The dose-dependent manner of CPRO increased the levels of A in $CCl_4$-treated groups, thus revealing their protective action.

Table 2 also presents the effect of CPRO on the kidney function markers of $CCl_4$-treated rats. Amounts of urea, creatinine and uric acid in the negative control (group 1) after 8 weeks of experiments were 25.3, 0.71 and 3.63 mg/dL, respectively. It could be seen that treatment with $CCl_4$ (group 2) resulted in an increase in urea, creatinine and uric acid levels (56.3, 1.85 and 7.93 mg/dL, respectively). On the other side, CPRO treatment caused a decrease in urea, creatinine and uric acid amounts.

The effect of CPRO on lipids profile of $CCl_4$-induced liver damage rats is shown in Fig. 1. Except for HDL-C, $CCl_4$ caused a significant increase in lipids parameters (TL, TC, TAG, LDL-C and VLDL-C). $CCl_4$ caused a decrease in the level of HDL-C after 8 weeks, while treatment with CPRO increased HDL-C levels. Levels of TL, TC, TAG, LDL-C and VLDL-C in group 2 (positive control) after 8 weeks, were 213.5, 292.6, 691.6, 119.5 and 58.5 mg/L, respectively. Levels of TL, TC, TAG, LDL-C and VLDL-C were significantly decreased to 165, 192, 565, 50.9 and 38.5 mg/L, respectively after 8 weeks with 200 mg/kg CPRO treatment.

MDA level (nmol/mg) was markedly greater in $CCl_4$-treated animals (group 2) after 8 weeks of the experiment (Table 3). An expected increase in the lipid oxidative markers in the $CCl_4$-treated rats, confirmed that oxidative damage was induced. Negative control (group 1) and groups treated with CPRO were characterized by low amounts of MDA. It could be noted that CPRO inhibited the elevation of MDA amounts as affected by $CCl_4$ treatment. On the other side, the GSH level was lower in $CCl_4$-treated animals (group 2). The GSH level was increased by CPRO treatment (groups 3 and 4) when compared with positive control. Administration of CPRO (groups 3 and 4) significantly ($p > 0.05$) increased the activity of GSH as compared with the $CCl_4$-treated group.

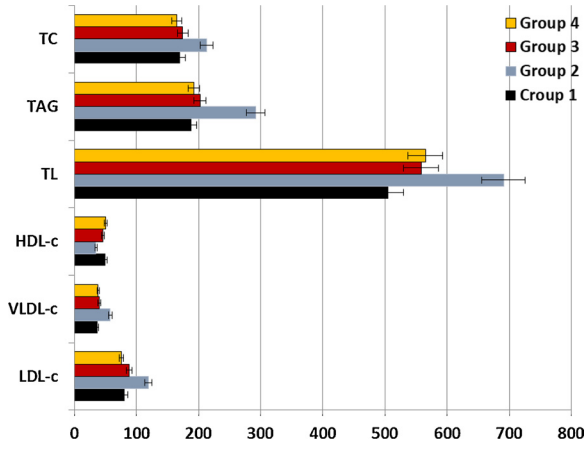


**Fig. 1.** Changes in lipids profile (mg/L) in $CCl_4$-induced injury in the rat as affected by CPRO. Error bars show the variations of three determinations in terms of standard deviation.

**Table 3**
Changes in antioxidant markers in $CCl_4$-induced injury in the rat as affected by CPRO.

| Group | Treatment | MDA (nmol/mg) | GSH (mmol/g) |
|---|---|---|---|
| 1 | Negative control (normal) | 3.35 ± 0.135$^{d}$ | 1.487 ± 0.025$^{a}$ |
| 2 | Positive control ($CCl_4$) | 9.252 ± 0.135$^{a}$ | 0.835 ± 0.025$^{b}$ |
| 3 | CPRO (100 mg/kg) + $CCl_4$ | 5.493 ± 0.135$^{b}$ | 1.279 ± 0.025$^{a}$ |
| 4 | CPRO (200 mg/kg) + $CCl_4$ | 4.213 ± 0.135$^{c}$ | 1.851 ± 0.025$^{a}$ |

$^{a, b, c, \cdots}$ *etc*There is no significant difference ($P > 0.05$) between any two means with the same letter in each column.

### 3.3. Liver histopathology

Fig. 2A-D presents the results of the hepatic histopathological examination. The histological observations supported the biochemical data. Microscopical examination of the liver of control animals revealed the normal histological structure of the liver. Liver sections from the negative control group exhibited normal cells with a well-preserved cytoplasm, lobular architecture, and well-defined nucleus and nucleoli (Fig. 2A). When compared with the normal liver tissues of control animals, liver tissue in $CCl_4$-treated rats revealed an extensive liver injury that characterized by moderate to severe hepatocellular degeneration and necrosis around the central vein, hepatic fibrosis, lipidosis and cholangiocyte hyperplasia (Fig. 2B). Liver of animals received $CCl_4$ exhibited severe fatty changes in the hepatocytes, the cytoplasm of hepatocytes showed clear vacuoles that squeezing their nuclei in one side giving signet ring shape. The portal blood vessels and hepatic sinusoids also showed severe congestion. Some sections revealed multiple areas of hemorrhage scattered all over the hepatic tissue. The hepatocytes suffered from various degrees of degeneration manifested by vacuolar and hydropic degeneration (Fig. 2B).

The histopathological hepatic lesions induced by $CCl_4$ were ameliorated in central lobular necrosis, hepatic fibrosis and hepatic lipidosis by CPRO treatment (Figs. 2C and D). In group 3, that administered 100 mg/kg CPRO, mild degeneration in the hepatocytes in the form of vacuolar degeneration and activation of von-Kupffer cells together with focal mononuclear aggregations in the portal areas were observed (Fig. 2C). Liver of group 4 that administered 200 mg/kg of CPRO, showed congestion of the central vein and mild congestion of the hepatic sinusoids (Fig. 2D).

## 4. Discussion

The liver, as an organ of metabolism and excretion, is endowed with the task of detoxification. Toxicants such as viruses, fungal products, bacterial metabolites, pollutants and chemotherapeutic factors could cause different liver disorders [54]. Hepatocellular carcinoma, fibrosis, cirrhosis and hepatitis are the most serious liver diseases. Hepatotoxins, such as acetaminophen, ethanol and $CCl_4$, induced liver injury that is linked with hepatocyte degeneration and cell death [30]. $CCl_4$ is a toxic agent used to initiate ROS production in organs of experimental animals, thus disturbing antioxidant defense system [4,6,55]. $CCl_4$, is used xenobiotic to cause chemical liver injury, wherein $CCl_4$ is metabolized by liver microsomal cytochrome $P_{450}$ to free radicals including trichloromethyl ($\cdot CCl_3$) and proxy trichloromethyl ($\cdot OOCCl_3$) radicals

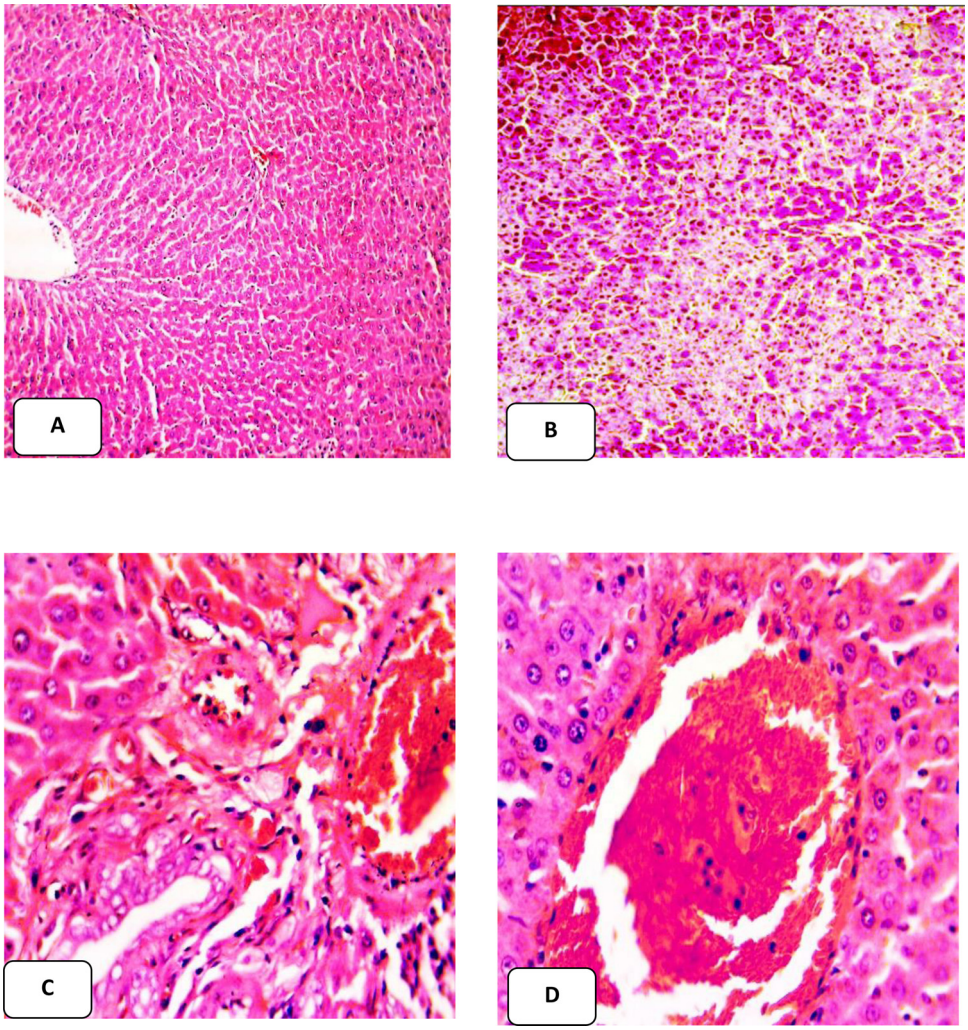


**Fig. 2.** Photomicrograph of liver from (A) control rats showing normal histopathological structure (H&E x 300), (B) rats received $CCl_4$ (100 mg/kg) showing severe fatty change in the hepatocytes (H&E x 300), (C) rats received $CCl_4$ (100 mg/kg) and CPRO (100 mg/kg) showing severe congestion of the portal blood vessels and mild hyperplasin of the epithelial liming of the bile duct (H&E x 800), (D) rats received $CCl_4$ 100 mg/kg and CPRO (200 mg/kg) showing thrombus formation in the portal blood vessels (H&E x 800).

[56,57]. Trichloromethyl might react with sulfhydryl groups (i.e., protein thiols and glutathione) and affect enzymes. Overproduction of trichloromethyl radicals initiate a membrane lipid oxidation, lead to liver steatosis, fibrosis, or cirrhosis. Superoxide and hydroxyl radicals were proved to link with the intoxication by $CCl_4$ [29]. Covalent binding of trichloromethyl-radicals to cell proteins is the first step in a chain of events that lead to membrane lipid oxidation and cell death.

There has been significant interest in the application of phenolics to treat liver diseases. An inverse relation between the consumption of phenolics-rich products and the risk of several diseases was reported [58]. Characterization of therapeutic traits of phenolics-rich plants and foods gained a great interest [59,60]. The antioxidant traits of phenolics and their ability to modulate the activity of enzymes were studied *in vitro* and believed to be a primary mechanism for their biological impacts. The question remains of whether these *in vitro* traits are relevant to protect against *in vivo* oxidative damage, wherein phenolic compounds exist at a low level upon the bioavailability and metabolism.

Antioxidant-rich plants were used to overpower oxidative stress in experimental animals. In the present study, CPRO was *in vivo* administered to animals to check the oil protective impact on $CCl_4$-induced liver toxicity. Researchers used liver enzymes as useful hallmarks of CCl4 liver toxicity [6,61–63]. Liver injury caused by $CCl_4$ elevated the levels of enzymes (AST, ALP, and ALT). In this work, serum markers were increased in the $CCl_4$-treated animals, hence proving the fact that structural integrity of hepatocytes was damaged as enzymes residing in the cytoplasm were released into the bloodstream. After $CCl_4$ induced toxicity, the elevation of antioxidant enzymes after CPRO administration to the animals resulted in a gross reduction of those enzymes.

$CCl_4$ increased lipid peroxidation, and influences blood biochemical parameters including liver enzymes, kidney function indicators, protein profile, lipid profiles and antioxidant markers. CPRO treatment prevented these harmful effects, indicating that CPRO could attenuate lipid oxidation induced by $CCl_4$. The health-promoting effect of CPRO was accompanied by partial prevention of GSH depletion in liver tissues. GSH, a non-enzymatic antioxidant, is involved in regulating the intracellular redox homeostasis and found in different cell types. GSH conjugation plays a critical role in the elimination of $CCl_4$ toxic metabolites [64]. Hepatic GSH represents an enzyme reserve of the liver that is responsible for reducing liver toxicity induced by $CCl_4$ metabolites. The level of GSH was reduced by $CCl_4$ treatment but upon CPRO administration, the increase in GSH amount was detected. The protection of CPRO on GSH reserves provide an action to remove the $CCl_4$ active metabolites and to scavenge radicals associated with lipid peroxidation. Our findings agree with Ali et al. [4] who reported the decrease of GSH levels in diseased conditions while uprising of the GSH levels upon treatment with *Parrotiopsis jacquemontiana* extract. Antioxidant

phytoconstituents might be the cause of CPRO protective traits.

The formation of peroxides by free radical derivatives of $CCl_4$ is one of the causes of $CCl_4$-induced liver damage. Therefore, the inhibition of the generation of free radicals is important in the protection against $CCl_4$-induced hepatotoxicity. The body could neutralize the radicals-induced damage by endogenous antioxidant enzymes (i.e., SOD and CAT). These enzymes constitute a defense against ROS. Lipid peroxidation, a ROS-mediated mechanism, has been implicated in the pathogenesis of liver injury and liver fibrogenesis in experimental animals. The non-dose dependent decrease in the hepatic hydroperoxide exhibited that CPRO treatment might protect against the liver lipid oxidation caused by $CCl_4$. Therefore, it is possible that the mechanism of CPRO hepatoprotection could be due to its antioxidative traits.

Impact of CPRO was studied by histological findings. Histopathological results are supported by the serum enzymes results. Liver histology might give a direct measure of liver damages induced by $CCl_4$ as well as ascribing CPRO as protective in ameliorating liver damages. Liver histology examination revealed the normal morphology of liver cells in control and vehicle groups with no anomaly recorded. Treatment with CPRO at different doses did not show an interruption of normal liver histo-architecture, whereas apparent liver damages were recorded in the $CCl_4$-treated animals. These protective impacts linked with CPRO could be due to its potent antioxidant potential displayed by inhibiting lipid peroxidation of the liver. Hepatocyte necrosis, fatty change, ballooning degeneration, hyaline degeneration, and infiltration of Kupffer cells and lymphocytes were prominent in the histopathological examinations. CPRO exhibited hepatoprotective traits in hepatic lipidosis, central lobular necrosis, and cholangiocyte hyperplasia in animals.

CPRO contains high amounts of essential health-promoting fatty acids. MUFA has been shown to reduce "bad" LDL-C and retain "good" HDL-C. CPRO contains also high levels of unsaponifiable matters including tools and phenolics. Oils rich in phenolics play a great role in preventing the harmful effects of many diseases. However, the bioavailability of phenolics was found to be higher when phenolic compounds are soluble in a lipid matrix than in an aqueous media [41,45]. Antioxidant effect of phenolics is due to their redox traits and is the result of antiradical traits, transition-metal-chelating activity and singlet-oxygen-quenching capacity [65]. Phenolic compounds show strong hepatoprotective impacts and may prevent inflammatory response, dyslipidemia, and mitochondrial oxidative damage of animals hepatocytes. Therefore, bioactive phytochemicals with high antioxidant potential, superior free radical-scavenging ability, and inhibition of oxidation are contributed to the hepatoprotective traits in animal models [30,66–70]. Phytochemical analysis of CPRO showed the presence of tools and phenolics, wherein the hepatoprotective traits of those constituents are well-demonstrated. From the obtained results, it could be said that CPRO exhibits antioxidant activities, which could have beneficial and health-promoting effects against oxidative liver injury induced by $CCl_4$.

## 5. Conclusion

CPRO is a promising oil, a rich source of bioactive phytonutrients (MUFA, PUFA, tocols and phenolics) and possessed hepatoprotective impacts against $CCl_4$-induced toxicity in animal models. Tocopherols and tocotrienols are effective scavengers of reactive nitrogen species (i.e., nitrogen dioxide, and peroxynitrite), and prevent DNA-bases nitration. CPRO could be successfully applied in different pharmaceutical applications. Looking forward to the clinical applications, CPRO might represent a possible therapeutic strategy against liver injury.

## Compliance with ethical standards

All animal experiments were performed in accordance with the acts of the international and institutional guidelines. These guidelines were in accordance with the internationally accepted principles for laboratory use and care.

## Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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