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Journal:	<i>Applied Physiology, Nutrition, and Metabolism</i>
Manuscript ID	apnm-2018-0246.R1
Manuscript Type:	Article
Date Submitted by the Author:	19-Jul-2018
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Keyword:	stress < stress, animal model(s) < research models, injury, diet, physiology, lipid metabolism
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

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Protective effect of *Cinnamomum zeylanicum* L. bark essential oil, on hepatic and renal toxicity induced by CCl₄ in rats

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Abbreviations

C. zeylanicum: *Cinnamomum zeylanicum*; CzEO : *C. zeylanicum* essential oil; ROS: reactive oxygen species; SOD: superoxide dismutase; CAT: catalase; GPX: glutathione peroxidase; DPPH: 1-Diphenyl-2-picrylhydrazyl; NBT: Nitroblue Tetrazolium; EDTA: Ethylenediaminetetraacetic acid; TBS: Tris Buffered Saline; TCA: Trichloroacetic Acid; BHT: Butylated hydroxytoluene; TBA: Thiobarbituric Acid; SD: Standard deviation; TRIS: trishydroxymethyl aminomethane; GSH: glutathione; DTNB: 5,5'-Dithio-bis(2-nitrobenzoic acid); TBARS: thiobarbituric acid reactive substances.

Draft

Abstract

The inner bark of cinnamon (*Cinnamomum zeylanicum* L.) is widely used as a spice. *Cinnamomum* plants are also a valuable sources of essential oil used for medicinal purposes. The present study aimed to investigate: the composition, the *in vitro* antioxidant activity of *C. zeylanicum* bark essential oil (CzEO) and its protective effects *in vivo* on CCl₄-induced hepatic and renal toxicity in rats. Groups of animals were pretreated for seven days with different concentrations of CzEO or controls and on day 7 a single dose of CCl₄ was used to induce oxidative stress in rats. Twenty-four hours after CCl₄ administration, the animals were sampled. In the controls, CCl₄ induced an increase of serum biochemical parameters and triggered oxidative stress in both liver and kidneys. CzEO (100 mg/kg) caused significant reductions in CCl₄-elevated levels of ALT, AST, ALP, LDH, γ GT, total cholesterol, triglycerides, LDL urea and creatinine and increased the level of HDL compared to the CCl₄ group. Moreover, pre-treatment with the CzEO at doses of 70 and 100 mg/kg BW to the rats treated with CCl₄ produced significant reductions in TBARS and PCO levels in liver and kidney tissues as compared to CCl₄ group. The formation of pathological hepatic and kidney lesions induced by the administration of CCl₄ was strongly prevented by CzEO at a dose of 100 mg/kg BW. Overall, this study suggests that administration of CzEO displayed high potential to quench free radicals and alleviate CCl₄-induced hepatorenal toxicity in rats.

Keywords: Animal model(s); Diet; Injury; Lipid metabolism; Physiology; Stress.

Introduction

Herbal medicines derived from plant extracts constitute an indispensable part of the traditional medicine and are being increasingly utilized to treat a wide variety of clinical diseases (**Gupta et al. 2004**). Antioxidants, industrial synthesized or natural occurring can protect the human body against the harmful effect of free radicals (**Lobo et al. 2010**). In this context, more attention needs to be paid to the protective effects of natural antioxidants of the herbal medicine against drug-induced toxicities (**Frei and Higdon 2003**). Cinnamon from the Lauraceae family is a notable plant in traditional and modern medicines; it is obtained from the bark of trees from the genus *Cinnamomum*. This herbal medicine has two main varieties; *Cinnamomum zeylanicum* (CZ) and *Cinnamomum cassia* (also known as *Cinnamomum aromaticum*/Chinese cinnamon). Cinnamon a well-known food additive for its taste is native to Sri Lanka and southern parts of India (**Ranasinghe et al. 2002; Paranagama et al. 2010**). The most important constituents of cinnamon are aromatic aldehyde 3-phenyl-2(E)-propenal, also called cinnamaldehyde, trans-cinnamaldehyde (Cin), and cinnamic acid (**Wang et al. 2007**). Throughout history, cinnamon has been believed to have several health benefits such as reducing triglyceride, LDL, total cholesterol, (**Blevins et al. 2007**) anti-inflammatory properties, anti-microbial activity, reducing cardiovascular disease, reducing blood glucose (**Mollazadeh and Hosseinzadeh 2016**), boosting cognitive activity, reducing risk of colonic cancer (**Ranasinghe et al. 2012**), anti-Parkinson (**Khasnavis and Pahan 2012**), anti-Alzheimer (**Malik et al. 2015**), anti-angiogenesis (**Hamidpour et al. 2015**), anti-HIV-1 activity (**Connell et al. 2016**), anti-yeast activity (**Brr and Mahmoud 2005**), anti-inflammatory action, anti-platelet aggregation, and improving blood circulation (**Chung et al. 2011**). One other example is Silymarin, a flavonolignan complex mixture from milk thistle plant, is already used for treatment of liver diseases, which we used in this study as reference. The hepatoprotective activity of silymarin acts by inducing liver cell regeneration and cell membrane stabilization to prevent hepatotoxic agents such as CCl₄ from entering hepatocytes (**Liu et al. 2012**).

Liver is the main organ for metabolism and detoxification and therefore the primary place where toxic substance have an impact. Several *in vitro* and animal models have been developed to study toxic effects and the model of CCl₄-induce liver damage in rats is well characterized. Carbon tetrachloride (CCl₄) is a compound that was before time used as a cleaning solvent in industry. Its industrial use has been largely abandoned due to adverse health effects such as hepatotoxicity (**Ogeturk et al. 2005; Jaramillo-Juarez et al. 2008**). CCl₄ induces the production of several types of reactive oxygen species (ROS), thereby causing liver injury (**Tada et al. 2003**). It is therefore considered as the experimental model of choice for liver injury (**Weiler-Normann et al. 2007**). **Abraham et al. (1999)** showed that the nephrotoxic effects of CCl₄ were also associated with free radical production. Accordingly, elimination of free radicals and prevention of lipid peroxidation have been targeted in prevention and treatment of hepatic and kidney damage (**Valko et al. 2007**).

From previous experimental and clinical studies performed on *C. zeylanicum* bark essential oil (CzEO), it seems that most of its pharmacological action is due to its antioxidant activity to trap free radicals and/or inhibit lipid peroxidation (**Singh et al. 2006**). Hence, in the present study, we report the chemical composition and antioxidant effects of essential oil of CzEO in several *in vitro* systems. Moreover, we focused on evaluating prevention of side effects by CzEO of liver and kidney damage in rats exposed to a single dose of CCl₄.

Materials and methods

Plant material

C. zeylanicum L. barks were acquired from a local market in Sfax (Tunisia). The collected barks were mixed. The samples were identified and authenticated by a senior botanist, Pr. Ferjani Ben Abdallah, at the Faculty of Science of Sfax, University of Sfax (Tunisia). A voucher specimen was deposited at the herbarium of the Faculty of Science of Sfax (Tunisia). The collected barks were air-

dried at room temperature (25°C) for 13 days in a well-shaded and naturally ventilated space. After drying, the samples were ground to a fine powder used for the extraction of essential oil.

Essential oil preparation

The oil extraction was obtained from 1 kg dry plant material by steam distillation during 3 h using a Clevenger-type apparatus. The aqueous phase was extracted with dichloromethane and dried with anhydrous sodium sulphate. Following filtration, the solvent is removed by pressure distillation reduced in rotary evaporator and pure oil was stored at 4 °C in obscurity until the beginning of CzEO analysis.

***C. zeylanicum* L. bark essential oil composition**

The isolated compounds from CzEO were analysed by GC-MS, using a Hewlett-Packard HP: 5890 series II. The fused HP-Innowax capillary column (polyethylene glycol, 30 m, 0.25 µm, ID, 0.25 mm film thickness) was coupled to a HP 5972A masse-selective detector (Hewlett-Packard, Palo Alto, CA, USA). The oven temperature was programmed from 50°C (1 min) to 250°C (5 min) at 7°C/min. The temperature of the injector port was held at 250°C, split: 1/100, the temperature of the detector was set at 280°C. The carrier gas was helium (99.995% purity), at a flow rate of 1.2 ml/min and the analysed sample volume was 2 µl. The mass spectrometer (MS) conditions were as follows: ionization voltage, 70 eV; ion source temperature, 150°C; electron ionization mass spectra were acquired over the mass range 50-550 m/z.

Component identification

The essential oil compounds were identified by comparing the mass spectra data available from the wiley 275 mass spectra libraries (software, D.03.00).

***In vitro* study**

Antioxidant activities of CzEO

The antioxidant activity of the CzEO was analyzed by two tests (DPPH radical-scavenging and Superoxide radical scavenging activity) and compared with the activity of silymarin, a bioactive extract isolated from *Silybum marianum* seeds (Asteraceae). Silymarin contains a 65-85% flavonolignans and approximately 20 % to 30 % chemically undefined fraction, comprising mostly polyphenolic compounds. Silymarin is used as a standard drug.

The free radical scavenging activity of CzEO was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay as specified by **Blois (1958)**. Silymarin was used as reference standard. The activity is given as % DPPH scavenging and calculated according to the following equation: % DPPH scavenging = $[(\text{control OD} - \text{sample OD}) / \text{control OD}] \times 100$. Each sample was analyzed six times. The inhibition of Nitro Blue Tetrazolium (NBT) reduction by photochemically generated $O_2^{\cdot -}$ was used to determine superoxide anion scavenging activity (**Yen and Chen 1995**). Superoxide anion was generated in a non-enzymatic system and determined by spectrophotometric measurement for the reduction of nitroblue tetrazolium. Silymarin was used as reference standard. The scavenging activity on superoxide anion generation was calculated as: % superoxide anion scavenging = $[(A_0 - A_1) / A_0] \times 100$

Where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of CzEO. Each sample was analyzed six times.

***In vivo* study**

Animals

A total of 60 male two-month-old *Wistar* albino rats weighing between 180 and 220 g were purchased from the Central Pharmacy (SIPHAT, Tunisia). The conditions of the animal house meet the agreed international standards (**Council of European Communities 1986**) and approved by the Ethical Committee of the Faculty of Sciences of Sfax.

All rats were free of major pathogens as described previously (**Klötting and Vogt, 1991**). The animals were housed at a 22 ± 3 °C temperature, $40 \pm 5\%$ humidity and a 12-h light-dark cycle. The animals had free access to commercial pellet diet (SICO, Sfax, Tunisia) and composed of casein (200 g/kg), DL-

methionine, corn oil (155 g/kg), cornstarch (393g/kg), sucrose (154 g/kg), cellulose (50 mg/kg), mineral mixture (35 g/kg), vitamin mixture (10 g/kg), and water *ad libitum*. The experimental protocols were conducted in accordance with the guide for the care and use of laboratory animals issued by the University of Sfax, Tunisia, and approved by the Committee of Animal Ethics (Protocol no. 94-1939).

Experimental design

One week after acclimatisation to laboratory conditions, 60 rats were randomly allocated into 6 experimental groups of ten animals each and treated for seven days as follow (**Mihailović et al. 2013**): (Figure 1).

Rats were killed twenty-four hours after vehicle or CCl₄ single injection. The animals in the different groups were killed by cervical dislocation to avoid stress conditions.

At the end of the experiment period (day 8), rats were sacrificed by cervical dislocation 24 h after the administration of CCl₄ and the trunk blood collected. The plasma was obtained by centrifugation (2500 x g, 15 min, 4°C) and stored at -20°C for biochemical assays. Liver and kidney were dissected, cleaned and weighed. Some samples were homogenized (1:2, w/v) in 50 mmol/l Tris buffer (pH 7.4) containing 150 mmol/l NaCl using an ultra-Turrax device. Some samples were rinsed, homogenized and centrifuged at 5000g for 25 min at 4°C and aliquots of supernatant were kept at -20°C until analyses. Other samples were fixed in 10% buffered formalin solution and embedded in paraffin for histological examination. For the biochemical and the histological experiments, samples (liver and kidney) were taken from ten rats in each group.

Biochemical assays

Biochemical markers in plasma

Plasma levels of (AST), (ALT), (ALP), (γ -GT), cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), creatinine and urea concentrations were measured

by autoanalyzer (Vitalab Flexor E, Diamond Diagnostics, Holliston, MA) using Biolabo diagnostic kits.

Protein quantification

The Protein contents were assayed according to method of **Lowry et al. (1951)** using bovine serum albumin (BSA) as a standard.

Evaluating lipid peroxidation levels

Lipid peroxidation concentrations in liver and kidney were assayed spectrophotometrically by measuring the formation of thiobarbituric acid reactive substances (TBARS) according to **Draper and Hadley (1990)**.

Evaluating protein carbonyl (PCO) levels

Protein carbonyl (PCO) concentrations were measured using the method of **Reznick and Packer (1994)**.

Evaluating enzymatic and non-enzymatic antioxidant levels in liver and kidney tissue

Changes in absorbance were monitored at the wavelength of 240 nm against the blank in 1 minute. The chemical reaction was triggered with the addition of hydrogen peroxide and then the enzyme activity (CAT) was calculated according to **Aebi (1984)**.

Superoxide dismutase (SOD) assay was carried out by the method of **Beyer and Fridovich (1987)**. The developed blue color in the reaction was monitored at the wavelength of 560 nm. Units of SOD activity were measured as the amount of enzyme required to inhibit the reduction of NBT (4-nitroblue tetrazolium chloride) by 50% and the activity was expressed as units/mg protein.

Glutathione peroxidase (GPx) activity was carried out by the procedure of **Flohé and Gunzler (1984)**. GPx catalyses the oxidation of reduced glutathione by cumene hydroperoxide. The absorbance was monitored at the wave length of 340 nm. Results were expressed as nmol of GSH/min/mg protein.

Glutathione (GSH) was carried out by the method of **Ellman (1959)** with slight modification by **Jollow et al. (1974)** based on the development of a yellow color when 5,5-dithiobis-2 nitro benzoic acid (DTNB) was added to compounds containing sulfhydryl groups. The absorbance was monitored at the wavelength of 412 nm after 10 min. GSH content was expressed as mg/mg of protein.

Histopathological studies

The liver and kidney tissues were fixed in 10% formalin for 48 h, then dehydrated in graduated ethanol (50-100%) and embedded in paraffin. Sections 5µm thick were made and stained with hematoxylin and eosin dye, then examined for histopathological changes under light microscopy (ZEISS, Axiolab) and fitted with Canon Power Shot camera (A640) to capture images for histological studies. All sections were evaluated to know about for the degree of liver and kidney injury.

Statistical analysis

Statistics were performed on SPSS software. Data were expressed as the mean $\pm$ SD and analyzed by one-way analysis of variance (ANOVA) followed by Fisher's post-hoc test (PLSD) for comparison between groups [treated groups (CCl₄ vs (Cont))] and [(CzEO + CCl₄) vs (CCl₄)]. Differences were considered significant at $p < 0.0$

Results

Chemical constitution of *C. zeylanicum* bark essential oil

Chemical composition of (CzEO) was assayed by GC/MS analysis. The identified compounds, their percentages as well as their retention indices are listed in Table 1. CzEO is a mixture with 16 compounds representing 98.44% of the total oil composition. Among the phytochemicals, the phenylpropanoid cinnamaldehyde was the most abundant (81.05%) followed by the monoterpene oxide 1, 8-cineole (3.56%), and the sesquiterpene α -copaene (3.29%).

Essential oil antioxidant activity

The 50% scavenging concentration on DPPH radical (IC₅₀) values of CzEO and silymarin was 0.36 ± 0.10 and 0.021 ± 0.21 mg/ml, respectively. Superoxide anion was also scavenged (Table 2). The

50% scavenging concentration NBT was calculated by linear regression analysis and was found, respectively, at 0.48 ± 0.11 and 0.037 ± 0.09 mg/ml for CzEO and silymarin.

The effect of CzEO on biochemical factors

The results observed after pre-treatment with CzEO or silymarin on various biochemical factors in the groups under study are given in Tables 3, 4 and 5.

A single injection of CCl_4 caused a significant increase in ALT, AST, ALP, LDH, γGT , total cholesterol, triglycerides, LDL urea and creatinine levels, while HDL level was decreased compared to controls untreated rats. Pre-treatment with CzEO (70 and 100 mg/kg) prior to CCl_4 injection prevented the rise of ALT, AST, ALP, LDH, γGT , total cholesterol, triglycerides, LDL urea and creatinine and reduce the drop of HDL compared to the CCl_4 -damaged rats.

When a dose of CzEO (100 mg/kg) given for 7 days this did have a significant effect on any of the parameters studied in these experiments. In the control experimental group: pre-treatment with silymarin (50 mg/kg) for 7 days protected the livers and kidney against the effect of a single injection of CCl_4 . The results are given in Tables 3, 4 and 5.

Oxidative damage markers in liver and kidney tissues

Comparison of oxidative stress markers (TBARS) and (PCO) in tissue of CCl_4 -treated rats with the control animals indicated a significant increase in TBARS (Fig. 2AB) and PCO (Fig. 3AB) levels after the induction of oxidative stress. Pre-treatment with the CzEO at doses of 70 and 100 mg/kg BW prevented dose dependent the increase of TBARS and PCO levels when compared to that in the CCl_4 group. Pre-treatment with CzEO (100 mg/kg) resulted in almost a full protection from oxidative damage (TBARS and PCO) and the effect was comparable to that of pre-treatment with silymarin (50 mg/kg BW). CzEO itself in a dose of 100 mg/kg BW did not result in any significant change in the TBARS and PCO levels in the animals compared to control/untreated rats.

Effects of CzEO on SOD, CAT, GPx and GSH levels

Results presented in Tables 6-7 revealed a decrease in liver and kidney of CAT, SOD, GPx and GSH concentrations in CCl₄-treated rats compared to untreated animals. In fact, the decrease in hepatic and kidney CAT, SOD, GPx and GSH concentrations as a result of CCl₄ administration were largely prevented in the CzEO and silymarin groups. Pre-treatment with the doses (70 and 100 mg/kg BW) of CzEO prevented dose dependent the decrease of CAT, SOD, GPx and GSH levels compared with the CCl₄ group. Pre-treatment with the dose of 100 mg/kg could prevent the decrease in the CAT, SOD, GPx and GSH levels compared to the CCl₄ group. Moreover, silymarin increased in the CAT, SOD, GPx and GSH levels in the damaged rats in pre-treatment group. It is worth noting that there were no significant differences in the CAT, SOD, GPx and GSH levels in the pretreated group with only CzEO (100 mg/kg BW) compared to control untreated rats.

Histopathological findings in livers and kidneys

Liver damage

From the histological point of view, liver from rats in the healthy control group showed a normal liver lobular architecture and hepatocyte structure (Fig. 4A). In contrast, the liver of CCl₄-treated group exhibited the most severe damage of all groups, where the liver sections in this group showed necrosis, fatty changes, cellular hypertrophy, infiltrated kupffer cells and lymphocyte, cirrhosis, and nuclear degeneration in some areas (Fig. 4B). The pathological hepatic lesions induced by the administration of CCl₄ were remarkably prevented by CzEO in a dose-dependent manner (Fig. 4D-E-F), which was almost similar to standard silymarin group (Fig 4C). The histopathological variations were graded and summarized in Table 8.

Kidney damage

The kidney section in the control and CzEO-treated rat kidneys revealed normal glomeruli and tubulo interstitial cells (Fig 5A-G). The histological outline of the kidney section of the CCl₄-treated group proved significant degenerative changes compared with the control rats (Fig 5B). The kidneys of

the CCl₄-treated animals showed histo-pathological damage; this group showed a significant varying glomerular and tubular degenerations (i.e. atrophy of the glomerular and dilatation of urinary space). The pathological kidney lesions induced by the administration of CCl₄ were remarkably prevented by CzEO in a dose-dependent manner (Fig. 5D-E-F) which was almost similar to standard silymarin group (Fig 5C). The histopathological variations were graded and summarized in Table 9.

An schematic overview of all the results is given in figure 6. Each parameter is scored semi-quantitatively relative to the control group. Increase is indicated with shades from yellow (low) to red (highest) and decrease with shades of blue (light blue is small and dark blue is strongest decrease). This shows that CCl₄ induces histological damage in liver and kidney and this is paralleled with increased release of liver enzymes into the serum and increased markers of oxidative damage in liver and kidney tissue. At the same time there is a reduction of enzymes that can counteract this oxidative stress. CzEO can prevent the negative effects of CCl₄ in a dose dependent manner, whereas CzEO does not have an effect on markers of tissue damage or oxidative stress. All observations that were made in this study are in good agreement.

Discussion

The major component of the CzEO is cinnamaldehyde (81.05%) followed by the monoterpene oxide 1,8-cineole (3.56%), and the sesquiterpene α -copaene (3.29%). Our findings are in accordance with previous studies of *C. zeylanicum* L. oils that also had cinnamaldehyde as their most considerable component (Mathew and Abraham 2006; Singh et al. 2006; Moarefian et al. 2011). Many factors including harvesting time of the aromatic plant, climatic and agronomic conditions, vegetative cycle stage, different age and segment of the plant, the plant part used, extraction processes and assay methods have been reported to contribute to discrepancies in the observed cinnamaldehyde concentrations of various cinnamomum essential oils (Geng et al. 2011). Moreover, cinnamaldehyde, found at relatively great concentration in the CzEO used in our study, have been related to subject anti-

inflammatory activity (**Tung et al. 2008**), making CzEO useful against oxidative damage of the liver and kidney after an intraperitoneal administration of CCl₄. Results of GC-MS analysis revealed that CzEO is rich in monoterpene phenols, especially cinnamaldehyde that has antioxidant properties (**Fu et al. 2008; Vuda et al. 2012**), this has to be executed when estimating different CzEO mixtures. Cinnamon bark oil was administered orally at the dose of 100 mg kg⁻¹ day⁻¹ for 7 days. As the toxicity studies suggest that dose of 100 mg kg⁻¹ day⁻¹ has no toxic effect (**Shah et al. 1998**), therefore, this dose was selected in this study. Also, one important difference between *C. cassia* and *C. zeylanicum* is their coumarin content (**Archer, 1988**). The levels of coumarin in *C. cassia* appear to be very high and pose health risks if consumed regularly in higher quantities.

In the present work, the hepatic histoarchitecture of the CCl₄-treated rats resulted large numbers of inflammatory cells such as lymphocytes along with hepatic sinusoidal inflammation, hepatocyte necrosis and devastating liver architecture. Moreover, the histological changes seen in the kidney of rats treated with CCl₄ were characterized by some nephrotoxic lesions, as evidenced by the glomerular and tubular necrosis and intratubular dilatation.

The histological finding of liver and kidney of the rats treated with the great dose of CzEO or silymarin showed improved hepatocellular and nephrocellular architecture with signs of recovery, indicating the protective effect of CzEO. In agreement with these results, the protective effect of essential oil of *C. zeylanicum* against carbon tetrachloride may be attributed to the presence of constituents of essential oil.

Our results demonstrated that injection of CCl₄ to rats causes serious liver damage which is spotted due to the rise in the serum levels of AST, ALT, ALP, LDH and γ GT. Deteriorate to liver cells changes their functional transition, induces membrane permeability, and leads to the leakage of enzymes into extracellular space (**Poli et al. 1987; Rolo et al. 2012**). Another indicator of damage to liver cells is lipid peroxidation (**Fernandez and West 2005**). In fact, lipid peroxidation shows the imbalance between the amount of free radicals and antioxidants in the body. That is, if the amount of

antioxidants in the body is less than the amount of oxidants, these extra oxidants can bind with body's critical compounds including the double bonds of membrane and cause damage (**Marimuthu et al. 2013**).

In addition, injection of CCl_4 caused a significant increase in the triglyceride, total cholesterol, and LDL levels and decrease in HDL level. Rise in the cholesterol concentrations might be due to the reduced esterification of fatty acids, inhibition of fatty acid β -oxidation, and diminished excretion of cellular lipids (**Sanzgiri et al. 1997**). CCl_4 induces the transfer of acetate into liver cells and leads to an increase in cholesterol production. It also increases the synthesis of fatty acids and triglyceride from acetate and initiates lipid esterification (**Weber et al. 2003**). The accretion of triglyceride in liver might occur due to the inhibition of lysosomal lipase activity and VLDL secretion (**Jaramillo-juárez et al. 2008**). Carbon tetrachloride, besides practicing its toxic effect on liver, also reportedly gets distributed at higher levels in the kidney than in the liver (**Achliya et al. 2004**). The mechanism of CCl_4 renal toxicity is almost the same as that of liver, but CCl_4 indicates a high affinity to the kidney cortex which contains cytochrome P-450 mainly (**Martins et al. 2008**). Due to CCl_4 hepatorenal injury, the transport function of hepatocytes and nephrotic cells gets disturbed resulting in the leakage of plasma membrane, thereby inducing a raised enzyme level in the serum (**Usta et al. 2013**). The injection of CCl_4 caused also renal toxicity detected by an increase of serum creatinine and urea (**AlYahya et al. 2013**). Pre-treatment of rats with CzEO before to CCl_4 exposure led to a protective effect against acute hepato and renotoxicity and oxidative stress, which was corroborated by the hepatic and kidney histopathological investigations. The stimulation of hepatic regeneration makes the liver more tolerant to damage induced by the carbon tetrachloride (**Saleem et al. 2015**). Pre-treatment with silymarin (50 mg/kg) or CzEO (100 mg/kg) significantly reduced the elevated levels of ALT, AST, ALP, LDH, γ GT, total cholesterol, triglycerides, LDL urea and creatinine and increased of HDL level as compared to CCl_4 group. The ethanolic extract of parsley leaves also revealed significant anti-inflammatory (**Anand et al. 2010**) and antioxidant (**Javed et al. 2012**) activities which may contribute to its hepatoprotective

action. The anti-inflammatory activity of *C. zeylanicum* L. bark essential oil could be partly explained by the presence of cinnamaldehyde (Szymonik-Lesiuk et al. 2003; Yadav et al. 1997).

In the present study, the increase in the TBARS and PCO levels and reduce activity of SOD, CAT, GPx and GSH observed in liver and kidney homogenate of CCl₄-intoxicated rats. The decreased level of GSH in liver and kidney may be due to NADPH reduction or GSH utilization in the exclusion of peroxides (Cheng et al. 2006). GSH-dependent enzymes afford a second line of protection as they primarily detoxify noxious by products generated by ROS (Yadav et al. 1997).

Pre-treatment with silymarin (50 mg/kg) or CzEO (100 mg/kg) resulted in almost a full protection from oxidative damage (TBARS and PCO) compared to the CCl₄ group. Moreover, pre-treatment with silymarin (50 mg/kg) or CzEO (100 mg/kg) significantly reduced the decrease in the CAT, SOD, GPx and GSH levels compared to the CCl₄ group.

They explained that the antioxidant present in the CzEO may help to trap ROS and peroxidation products and offer protection to enzymes. This hypothesis is supported by the recent finding on the *in vitro* antiradical and antioxidative activities of CzEO (Ervina et al. 2016). Chen et al. (2015) showed that cinnamaldehyde was the main compounds origin for antioxidant activity of CzEO.

In fact, the phytochemical composition of essential oil of *C. zeylanicum* showed the great content of terpenic derivatives (such as carvacrol, linalool, geraniol, citronellol, menthone, terpinen-4-ol, and α -terpineol) and phenylpropanoid compounds (eugenol and cinnamaldehyde). Previous studies revealed that cinnamaldehyde was the main compounds origin for antioxidant activity of CzEO (Chen et al. 2015). These compounds could interact with the reactive oxygen species ROS induced by carbon tetrachloride which induced aggressive oxidants (Ervina et al. 2016). Therefore, our results showed that the essential of *C. zeylanicum* exhibited an excellent guardable effect and may be well-respected as a useful source of cellular defense agent in liver and kidney tissues against carbon tetrachloride.

Conclusions

Overall, this study indicates that CzEO has a preventive effect against CCl₄-induced hepatotoxicity and nephrotoxicity in rats. This hepatorenal preventive effect may be attributed to its ability to scavenge free radicals and to inhibit ROS accumulation. From a clinical perspective it could be beneficial when natural ingredients like CzEO can be supplemented in the diet and in that way increase the protection against toxic exposure. For this more clinical studies are needed to elucidate the efficacy of this product in humans.

Acknowledgments

The present work was supported by DGRST grant (Direction Generale de la Recherche Scientifique et Technique-Tunisie: Appui a la Recherche Universitaire de base UR/13 ES-73).

Conflicts of interest

The authors declare that there is no conflict of interest

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Table 1. Chemical composition (%) of barks essential oil from *C.zeylanicum* carried out by GC/MS analysis

Peak	Compounds	Retention Time (min)	Percentage (%)
1	Camphene	10.49	0.7
2	α -Pinene	14.85	2.19
3	Δ -terpinene	15.60	0.15
4	1,8-Cineole	16.02	3.56
5	Linalool	19.65	0.62
6	Isobutanol	20.39	0.12
7	camphenilol	22.67	0.01
8	Terpinen-4-ol	24.06	0.46
9	Borneol	24.94	0.62
10	4,7-dimethylundecane	27.83	0.48
11	α -copaene	28.53	3.29
12	Endobornylacetate	30.94	1.23
13	Geranylacetate	31.09	0.72
14	β -caryophyllene	34.67	1.35
15	Cinnamaldehyde	37.61	81.05
16	1 s,cis-calamenene	39.24	1.7
			Total (%)= 98.44

Table 2. CzEO effects and positive controls on the *in vitro* free radical (DPPH and superoxide). Values are represented as mean $\pm$ SEM of six different experiments.

Parameters	CzEO	Silymarin
50% scavenging concentration (mg/ml) on DPPH radical	0.36 $\pm$ 0.10*	0.021 $\pm$ 0.21
50% scavenging concentration (mg/mL) on superoxide anion	0.48 $\pm$ 0.11*	0.037 $\pm$ 0.09

* p < 0.05 versus silymarin.

Table 3. Effects of CCl₄, CzEO and their combination CzEO/CCl₄ on hepatic markers in serum of control and experimental rats

Parameters and treatment	AST (U/L)	ALT (U/L)	ALP (U/L)	LDH (U/L)	γ -GT (U/L)
Control	141.779 $\pm$ 1.216	41.034 $\pm$ 1.523	126.172 $\pm$ 2.200	16.094 $\pm$ 0.546	1.952 $\pm$ 0.350
CCl ₄	501.306 $\pm$ 5.176***	154.454 $\pm$ 3.573* **	194.130 $\pm$ 8.650* **	35.806 $\pm$ 0.622***	4.516 $\pm$ 0.155* **
SL/CCl ₄	168.054 $\pm$ 2.310####/ @@@	57.506 $\pm$ 6.628###/ /@	134.144 $\pm$ 5.167# #	23.664 $\pm$ 0.814####/ @@@	2.476 $\pm$ 0.099# ##
^a CzEO/C Cl ₄	333.688 $\pm$ 35.737##/ /@@@	135.218 $\pm$ 5.512#/ @@@@	163.222 $\pm$ 4.058#/ @@@@	29.660 $\pm$ 1.379###/ @@@@	3.400 $\pm$ 0.249# #/@@
^b CzEO/C Cl ₄	193.876 $\pm$ 7.130####/ @@@	65.517 $\pm$ 3.917###/ /@@	142.784 $\pm$ 5.286# ##/@	27.937 $\pm$ 0.659####/ @@@@	2.610 $\pm$ 0.207# ##
^b CzEO	137.806 $\pm$ 2.510	40.634 $\pm$ 3.475	116.352 $\pm$ 7.255	15.594 $\pm$ 0.772	1.912 $\pm$ 0.240

AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; LDH: lactate dehydrogenase; γ GT: gamma glutamyltransferase.

^aCzEO (70 mg/kg BW), ^bCzEO (100 mg/kg BW), SL: Silymarin (50 mg/kg BW).

Values are mean $\pm$ SEM for ten rats in each group.

@ p < 0.05, @@ p < 0.01, @@@ p < 0.001, (CzEO), (CzEO/CCl₄) and (SL/CCl₄) treated groups vs control group;

** p < 0.01, *** p < 0.001, (CCl₄) vs control group;

p < 0.05, ## p < 0.01, ### p < 0.001, (CCl₄/CzEO) and (CCl₄/SL) groups vs (CCl₄) group.

Table 4. Effects of CCl₄, CzEO and their combination CzEO/CCl₄ on lipid profile in serum of control and experimental rats

Treatment and parameters	T-Cholesterol (mmol/l)	T-Triglycerides (mmol/l)	HDL (mmol/L)	LDL (mmol/l)
Control	2.163±0.053	1.553±0.054	2.413±0.023	0.213±0.018
CCl₄	2.750±0.028 ^{***}	2.576±0.185 ^{***}	1.446±0.074 ^{***}	0.893±0.047 ^{***}
SL/CCl₄	2.220±0.037 ^{###}	1.610±0.045 ^{##}	2.240±0.030 ^{###/ @}	0.263±0.021 ^{###}
^aCzEO/CCl₄	2.376±0.076 [#]	1.863±0.072 ^{#/ @}	1.840±0.078 ^{#/ @@}	0.686±0.048 ^{###/ @@}
^bCzEO/CCl₄	2.293±0.026 ^{###}	1.660±0.057 ^{##}	2.176±0.038 ^{###/ @@}	0.320±0.023 ^{###/ @}
^bCzEO	2.110±0.258	1.563±0.046	2.216±0.099	0.180±0.030

TC: Total cholesterol (mmol/l), **HDL:** high densitylipoprotein (mmol/l), **TG:**triglyceride (mmol/l), **LDL:**lowdensitylipoprotein (mmol/l). **^aCzEO** (70 mg/kg BW), **^bCzEO** (100 mg/kg BW), **SL:** Silymarin (50 mg/kg BW).

Values are mean ± SEM for ten rats in each group.

@ p<0.05, @@ p < 0.01, @@@ p < 0.001, (CzEO), (CzEO/CCl₄) and (SL/CCl₄) treated groups vs control group;

** p< 0.01, *** p < 0.001, (CCl₄) vs control group;

#p< 0.05, ##p < 0.01, ### p < 0.001, (CCl₄/CzEO) and (CCl₄/SL) groups vs (CCl₄) group.

Table 5. Effects of CCl₄, CzEO and their combination CzEO/CCl₄ on kidney markers in serum of control and experimental rats

Treatment	Urea (mmol/l)	Creatinine (µmol /l)
Control	5.984±0.660	9.150±0.351
CCl₄	13.410±1.381 ^{**}	10.294±0.372 ^{**}
SL/CCl₄	6.516±0.212 ^{##}	8.644±0.251 ^{##}
^aCzEO/CCl₄	8.692±0.540 ^{#/ @}	9.210±0.126 [#]
^bCzEO/CCl₄	6.838±0.410 ^{##}	8.692±0.180 ^{##}
^bCzEO	5.144±0.389	9.068±0.221

^aCzEO (70 mg/kg BW), **^bCzEO**(100 mg/kg BW), **SL:** Silymarin (50 mg/kg BW).

Values are mean ± SEM for ten rats in each group.

@ p<0.05, @@ p < 0.01, @@@ p < 0.001, (CzEO), (CzEO/CCl₄) and (SL/CCl₄) treated groups vs control group;

** p< 0.01, *** p < 0.001, (CCl₄) vs control group;

#p< 0.05, ##p < 0.01, ### p < 0.001, (CCl₄/CzEO) and (CCl₄/SL) groups vs (CCl₄) group.

Table 6. Effects of CCl₄, CzEO and their combination CzEO/CCl₄ on the activities of enzymatic and non-enzymatic antioxidants in kidney of control and experimental rats

Parameters and treatment	SOD ¹	CAT ²	GPx ³	GSH ⁴
Control	13.049±0.156	11.165±0.305	3.847±0.336	2.452±0.164
CCl ₄	10.188±0.452***	8.308±0.248***	2.043±0.214**	1.512±0.024***
SL/CCl ₄	12.650±0.167##	10.144±0.206###/@	3.618±0.093##	2.346±0.147###
^a CzEO/CCl ₄	11.503±0.187#/@@@	9.303±0.290#/@@	3.162±0.262#	1.668±0.059#/@
^b CzEO/CCl ₄	12.488±0.208##	10.143±0.290###/@	3.749±0.108##	2.436±0.151###
^b CzEO	12.734±0.243	10.994±0.299	3.558±0.292	2.488±0.086

¹: Units/mg protein; ²: μmol H₂O₂/min/mg protein; ³: μmol GSH/min/mg protein; ⁴: nmol/mg protein.

^aCzEO (70 mg/kg BW), ^bCzEO (100 mg/kg BW), SL: Silymarin (50 mg/kg BW).

Values are mean ± SEM for ten rats in each group.

@ p<0.05, @@ p < 0.01, @@@ p < 0.001, (CzEO), (CzEO/CCl₄) and (SL/CCl₄) treated groups vs control group;

** p< 0.01, *** p < 0.001, (CCl₄) vs control group; # p < 0.05, ## p < 0.01,

p< 0.001, (CCl₄/CzEO) and (CCl₄/SL) groups vs (CCl₄) group.

Table 7. Effects of CCl₄, CzEO and their combination CzEO/CCl₄ on the activities of enzymatic and non-enzymatic antioxidants in liver of control and experimental rats

Parameters and treatment	SOD ¹	CAT ²	GPx ³	GSH ⁴
Control	18.049±0.117	15.453±0.300	11.026±0.429	3.302±0.119
CCl ₄	15.746±0.383***	13.139±0.281***	8.433±0.322**	1.826±0.080***
SL/CCl ₄	17.829±0.460##	15.381±0.243###	10.511±0.158###	3.036±0.140###
^a CzEO/CCl ₄	17.340±0.288#/@	14.586±0.449#	9.547±0.277#/@	2.360±0.149#/@
^b CzEO/CCl ₄	17.671±0.370##	15.004±0.276###	10.307±0.225###	2.964±0.085###
^b CzEO	15.833±0.319	15.256±0.438	10.538±0.364	3.264±0.053

¹: Units/mg protein; ²: μmol H₂O₂/min/mg protein; ³: μmol GSH/min/mg protein; ⁴: nmol/mg protein.

^aCzEO (70 mg/kg BW), ^bCzEO (100 mg/kg BW), SL: Silymarin (50 mg/kg BW).

Values are mean ± SEM for ten rats in each group.

@ p<0.05, @@ p < 0.01, @@@ p < 0.001, (CzEO), (CzEO/CCl₄) and (SL/CCl₄) treated groups vs control group;

** p< 0.01, *** p < 0.001, (CCl₄) vs control group;

p< 0.05, ## p < 0.01, ### p < 0.001, (CCl₄/CzEO) and (CCl₄/SL) groups vs (CCl₄) group.

Table 8. Grades of the histopathological changes in the liver sections of rats CzEO, CCl₄, or CzEO+CCl₄.

Parameters	Control CCl ₄	SL/CCl ₄ CzEO ^a /CCl ₄ CzEO ^b /CCl ₄ CzEO ^b				
Inflammatory cells	-	+++	+	++	+	-
Cellular necrosis	-	+++	+	++	+	-

Scoring was done as follows: none (-), mild (+), moderate (++) and severe (+++).

^aCzEO (70 mg/kg BW), ^bCzEO (100 mg/kg BW), SL: Silymarin (50 mg/kg BW).

Table 9. Grades of the histopathological changes in the kidney sections of rats CzEO, CCl₄, or CzEO+CCl₄.

Parameters	Control CCl ₄	SL/CCl ₄ CzEO ^a /CCl ₄ CzEO ^b /CCl ₄ CzEO ^b				
Glomerular degenerations	-	+++	+	++	+	-
Tubular degenerations	-	+++	+	++	+	-

Scoring was done as follows: none (-), mild (+), moderate (++) and severe (+++).

^aCzEO (70 mg/kg BW), ^bCzEO (100 mg/kg BW), SL: Silymarin (50 mg/kg BW).

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Figures captions

Figure 1. Experimental protocol of control and experimental rats.

Figure 2A. Effects of CCl₄, CzEO and their combinations CzEO/CCl₄ on hepatic TBARS of control (Con) and experimental rats. Con, control group; mod, CCl₄-model group; SL/CCl₄, silymarin 50 mg/kg + CCl₄; CzEO/CCl₄ 70 mg/kg + CCl₄ group; CzEO/CCl₄ 100 mg/kg + CCl₄ group; CzEO 100 mg/kg.

Values are mean ± SEM for ten rats in each group.

@ p < 0.05, @@ p < 0.01, @@@ p < 0.001, (CzEO), (CzEO/CCl₄) and (SL/CCl₄) treated groups vs control group;

** p < 0.01, *** p < 0.001, (CCl₄) vs control group;

p < 0.05, ## p < 0.01, ### p < 0.001, (CCl₄/CzEO) and (CCl₄/SL) groups vs (CCl₄) group.

Figure 2B. Effects of CCl₄, CzEO and their combinations CzEO/CCl₄ on kidney TBARS of control (Con) and experimental rats. Con, control group; mod, CCl₄-model group; SL/CCl₄, silymarin 50 mg/kg + CCl₄; CzEO/CCl₄ 70 mg/kg + CCl₄ group; CzEO/CCl₄ 100 mg/kg + CCl₄ group; CzEO 100 mg/kg.

Values are mean ± SEM for ten rats in each group.

@ p < 0.05, @@ p < 0.01, @@@ p < 0.001, (CzEO), (CzEO/CCl₄) and (SL/CCl₄) treated groups vs control group;

** p < 0.01, *** p < 0.001, (CCl₄) vs control group;

p < 0.05, ## p < 0.01, ### p < 0.001, (CCl₄/CzEO) and (CCl₄/SL) groups vs (CCl₄) group.

Figure 3A. Effects of CCl₄, CzEO and their combinations CzEO/CCl₄ on hepatic protein carbonyls (PCO) of control (Con) and experimental rats. Con, control group; mod, CCl₄-model group; SL/CCl₄, silymarin 50 mg/kg + CCl₄; CzEO/CCl₄ 70 mg/kg + CCl₄ group; CzEO/CCl₄ 100 mg/kg + CCl₄ group; CzEO 100 mg/kg.

Values are mean ± SEM for ten rats in each group.

@ p < 0.05, @@ p < 0.01, @@@ p < 0.001, (CzEO), (CzEO/CCl₄) and (SL/CCl₄) treated groups vs control group;

** p < 0.01, *** p < 0.001, (CCl₄) vs control group;

p < 0.05, ## p < 0.01, ### p < 0.001, (CCl₄/CzEO) and (CCl₄/SL) groups vs (CCl₄) group.

Figure 3B. Effects of CCl₄, CzEO and their combinations CzEO/CCl₄ on kidney protein carbonyls (PCO) of control (Con) and experimental rats. Con, control group; mod, CCl₄-model group; SL/CCl₄, silymarin 50 mg/kg + CCl₄; CzEO/CCl₄ 70 mg/kg + CCl₄ group; CzEO/CCl₄ 100 mg/kg + CCl₄ group; CzEO 100 mg/kg.

Values are mean ± SEM for ten rats in each group.

@ p < 0.05, @@ p < 0.01, @@@ p < 0.001, (CzEO), (CzEO/CCl₄) and (SL/CCl₄) treated groups vs control group;

** p < 0.01, *** p < 0.001, (CCl₄) vs control group;

p < 0.05, ## p < 0.01, ### p < 0.001, (CCl₄/CzEO) and (CCl₄/SL) groups vs (CCl₄) group.

Figure 4. Effect of *C. zeylanicum* L. bark essential oil (CzEO) on CCl₄-induced liver damage. (A) Control group; (B) CCl₄-model group showing marked inflammatory cells, necrosis and

reduced lesions of necrosis; (C) Silymarin 50 mg/kg + CCl₄ group; (D) CzEO 70 mg/kg + CCl₄ group; (E) CzEO 100 mg/kg + CCl₄ group; (F) CzEO 100 mg/kg group. Hematoxylin/eosin staining; magnification $\times 400$.

$\Rightarrow$: Marked inflammatory cells; $\blacktriangledown$: Necrosis cells; $\quad$: Reduced lesions of necrosis

Figure 5. Effect of *C. zeylanicum* L. bark essential oil (CzEO) on CCl₄-induced kidney damage. (A) Control group; (B) CCl₄-model group showing some nephrotoxic lesions, as evidenced by the glomerular and tubular necrosis; (C) Silymarin 50 mg/kg + CCl₄ group; (D); CzEO 70 mg/kg + CCl₄ group; (E) CzEO 100 mg/kg + CCl₄ group; (G) MpEO 100 mg/kg group. Hematoxylin/eosin staining; magnification 100 $\times$ and 400 $\times$.

$\Rightarrow$: glomerular necrosis ; $\blacktriangledown$: necrosis in epithelial cells of the proximal tubules,
 $\downarrow$: Intratubular dilatation

Figure 6. An schematic overview of all the results.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

SERUM											
	AST	ALT	ALP	LDH	γ -GT	T-CH	T-TG	HDL	LDL	Urea	Creatinine
Cont	0	0	0	0	0	0	0	0	0	0	0
CCl ₄	3	3	2	2	3	2	2	-2	3	2	2
SL/CCl ₄	1	1	0	1	1	0	0	0	0	0	0
CzEO ^a /CCl ₄	2	3	2	1	2	1	1	-1	2	1	1
CzEO ^b /CCl ₄	1	1	1	1	1	1	0	0	1	0	0

HISTOLOGY										LIVER	
	Inflammatory cells		Necrosis	TBARS	PCO	SOD	CAT	GPx	GSH		
Cont	0	0	0	0	0	0	0	0	0		
CCl ₄	3	3	2	-2	-2	-2	-2	-2	-2		
SL/CCl ₄	1	1	1	-1	-1	0	-1	0	0		
CzEO ^a /CCl ₄	2	2	2	-2	-2	-2	-2	-2	-2		
CzEO ^b /CCl ₄	1	1	1	-1	-1	-1	-1	-1	-1		

HISTOLOGY										KIDNEY	
	Glomerular necrosis		Tubular necrosis	TBARS	PCO	SOD	CAT	GPx	GSH		
Cont	0	0	0	0	0	0	0	0	0		
CCl ₄	3	3	2	2	-2	-2	-2	-2	-2		
SL/CCl ₄	1	1	1	1	-1	0	-1	0	0		
CzEO ^a /CCl ₄	2	2	2	2	-2	-2	-2	-2	-2		
CzEO ^b /CCl ₄	1	1	1	1	-1	-1	-1	-1	-1		

Strong increase	
Medium increase	
Small increase	
Not significant	
Small decrease	
Medium decrease	
Large decrease	

Figure 6