



Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

journal homepage: www.elsevier.com/locate/apjtd



Document heading

doi:10.1016/S2222-1808(13)60070-5

© 2013 by the Asian Pacific Journal of Tropical Disease. All rights reserved.

Protective effects of *Lactuca sativa* ethanolic extract on carbon tetrachloride induced oxidative damage in rats

Hefnawy Taha M. Hefnawy¹, Mohamed Fawzy Ramadan^{1,2*}¹Agricultural Biochemistry Department, Faculty of Agriculture, Zagazig University, Zagazig 44519, Egypt²Institute of Scientific Research and Revival of Islamic Culture, Umm Al-Qura University, Makkah, Kingdom of Saudi Arabia

PEER REVIEW

Peer reviewer

Dr. Ali H. Amin, Faculty of Science, Zoology Department, Mansoura University, Mansuora, Egypt.

Department of Physiology, Hypertension and Renal Center of Excellence, Tulane University, 1430 Tulane Ave, New Orleans, LA 70112, USA.

Tel: +20-50-2223786

Fax: +20-50-2246781

E-mail: alihamin@gmail.com

Comments

This is a promising research work in which authors have demonstrated the hepatoprotective activity of *L. sativa* in CCl₄-induced liver damage in rats. The activity was assessed based on biochemical parameters, antioxidant enzyme levels in liver homogenate and histopathological observations. *L. sativa* was found to be a promising hepatoprotective agent in CCl₄ rat models.

Details on Page 284

ABSTRACT

Objective: To study the protective effects of the ethanolic extract of lettuce (*Lactuca sativa* L. var. longifolia) leaves against the toxicity caused by carbon tetrachloride (CCl₄) in reproductive system of rats.

Methods: Lettuce leaves were dried and extracted with ethanol (plant: solvent, 1:10, w/v). The extract was filtered and evaporated to yield dried lettuce extract. Animals were divided into seven groups and treated with CCl₄ and different concentrations of lettuce extract. At the end of the experimental period, the animals were sacrificed and blood was collected and centrifuged for serum separation. Body weights, testis size, histopathology of testis and liver, catalase (CAT) activity, superoxide dismutase (SOD) activity, peroxidase (POD) activity, reduced glutathione (GSH), glutathione peroxidase activity (GSH-Px), thiobarbituric acid reactive substances (TBARS), nitrite level, and serum hormones were determined.

Results: Oxidative stress induced by CCl₄ (2 mL/kg body weight) in rat decreases the increase in body weight and relative testis weight. It also markedly increases the level of TBARS and nitrites along with corresponding decrease in reduced glutathione and various antioxidant enzymes in testis (*i.e.*, CAT, POD, SOD and GSH-Px). Serum level of testosterone, luteinizing hormone and follicle stimulating hormone was decreased while estradiol and prolactin were increased during CCl₄ treatment. Histopathology of CCl₄-treated rats indicated the partial degeneration of germ and leydig cells along with deformities in spermatogenesis. Supplementation of lettuce extract (100, 150, 200 mg/kg body weight orally) once a week for 10 weeks results in decrease of TBARS and nitrite, while increase in antioxidant enzymes; CAT, POD, SOD, GSH-Px and GSH contents. Serum level of testosterone, luteinizing hormone, follicle stimulating hormone, estradiol, prolactin, histology, body weight and relative testis weight was also concomitantly restored to near normal level by lettuce extract supplementation to CCl₄-intoxicated rat.

Conclusions: The results clearly demonstrate that lettuce extract treatment augments the antioxidants defense mechanism against CCl₄-induced toxicity and provides evidence that it may have a therapeutic role in free radical mediated diseases.

KEYWORDS

Lettuce, *Lactuca sativa*, CCl₄, Superoxide dismutase, TBARS, Testosterone, Estradiol, Rats

1. Introduction

Liver is the first organ to metabolize foreign compounds and hence it is susceptible to many different diseases. Some

are rare but some diseases such as hepatitis, cirrhosis, alcohol-related disorders and liver cancer are common. A major cause of these disorders is due to exposure to different environmental pollutants and xenobiotics *e.g.*, paracetamol,

*Corresponding author: Mohamed Fawzy Ramadan, Associate Professor, Biochemistry Department, Faculty of Agriculture, Zagazig University, Zagazig 44519, Zagazig, Egypt.
Tel: +20-055-2320282, 002-055-2313940
Fax: +20-055 2287567, 002-055-2345452
E-mail: hassanienmohamed@yahoo.com or mframadan@zu.edu.eg
Foundation Project: Supported by Faculty of Agriculture, Zagazig University (Project Number 34-2012).

Article history:

Received 27 May 2013

Received in revised form 3 Jun, 2nd revised form 9 Jun, 3rd revised form 19 Jun 2013

Accepted 10 Jul 2013

Available online 28 Aug 2013

carbon tetrachloride, thioacetamide, alcohol, *etc.* These toxicants mainly damage liver by producing reactive oxygen species (ROS)[1]. The liver plays a crucial role in metabolisms of endogenous and exogenous substances, and the hepatic injury is associated with distortion of these functions. Liver function is generally impaired by xenobiotics or infections. Chronic or excessive exposure of xenobiotics is finally terminated to cirrhosis or malignant lesions in untreated cases. Nowadays, many people are suffering from hepatic damage induced by alcohol, chemicals and infections. Thus, acute and chronic liver diseases continue to be serious health problems in the world. Some natural compounds, such as silymarin and glycyrrhizin are demonstrated to have a protective role against liver diseases[2–5]. Hence, there has been considerable interest in role of naturally originated agents for the treatment of liver diseases.

Reactive oxygen free radicals have been known to produce tissue injury through covalent binding and lipid peroxidation and have been shown to augment fibrosis as seen from increased collagen synthesis[6,7]. Scavenging of free radicals by antioxidants could reduce the fibrosis process in the tissues[8,9]. Free radicals may also be a contributory factor in a progressive decline in the function of immune system[10]. Cooperative defense systems that protect body from free radical damage include the antioxidant nutrients and enzymes. The antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Their role as protective enzymes is well-known and has been investigated extensively with *in vivo* models.

Carbon tetrachloride (CCl₄), an industrial solvent, is an extensively used xenobiotic to induce chemical liver injury. CCl₄ is metabolized by hepatic microsomal cytochrome p450 to trichloromethyl-free radical. Trichloromethyl, hepatotoxic toxic metabolite of CCl₄, can react with sulfhydryl groups (glutathione and protein thiols) and antioxidant enzymes such as CAT and SOD. Over production of trichloromethyl-free radicals initiate a membrane lipid peroxidation, eventually leading to various liver pathological processes, such as steatosis, fibrosis or cirrhosis. The studies using antioxidants indicated the role of oxidative stress in CCl₄-induced liver injury[2,11]. Furthermore, some studies revealed that natural products, containing antioxidant, protect the liver against lipid peroxidation and impairment in antioxidant status induced by CCl₄[3,12]. These free radicals such as trichloromethyl radical (.CCl₃) and proxy trichloromethyl radical (.OCCl₃) were metabolized products derived from CCl₄ by cytochrome P450E1[13,14].

Epidemiological studies have suggested an inverse relation between the consumption of polyphenol-rich foods and beverages and the risk of degenerative diseases, particularly cancers and cardiovascular diseases[15]. In this regard, there has been a great deal of interest in the screening and characterization of novel potential therapeutic of compounds and polyphenol-rich extracts from foods and medicinal plants[16]. The antioxidant properties of polyphenols and their ability to modulate the activity of various enzymes have been

demonstrated in *in vitro* studies and are believed to be a primary mechanism for their biological effects[17]. However, the question remains that whether these properties demonstrated in *in vitro* studies are relevant to protect against oxidative damage *in vivo*, where polyphenols are at a very low concentrations depending on bioavailability and metabolism.

Fresh vegetables and fruits are associated with lower risk of cancer and cardiovascular diseases. Epidemiological studies have also demonstrated the relationship between dietary habits and disease risk. *Lactuca sativa* var *longifolia* (*L. sativa*) called romaine lettuce or cos lettuce, belongs to the botanical family of Asteraceae. As a healthier foods, lettuce is an important leafy vegetable that is consumed fresh or in salads[18–21]. Lettuce varieties have been investigated and reported to contain phenolic compounds with antioxidant activities[22]. Studies have shown the health impacts of lettuce in preventing cardiovascular diseases in rats and humans[23,24]. Anticonvulsant and sedative-hypnotic effects have been mentioned for the leaves of this plant[25]. Sayyah *et al.* reported that seeds extract had analgesic and anti-inflammatory activity in rats[26]. Lettuce methanol extract has high phenolic contents and shows strong radical scavenging activity. It was effective against some Gram negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Serratia marcescens*, *Acinetobacter baumannii*) and Gram positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium* and *Corynebacterium* spp.). Moreover, methanol and aqueous extracts exhibited antiviral activity against HCMV and Cox-B3 viruses[27]. The health-promoting benefits of lettuce have been attributed to the presence of vitamin C, phenolics and fibre[19,23,28]. Llorach *et al.* recently identified quercetin and luteolin rhamnosyl-hexosides in lettuce[22].

An experiment has been designed on rat model to evaluate the protective effects of the ethanolic extract of *L. sativa* against the toxicity caused by CCl₄ in reproductive system of rats.

2. Materials and methods

2.1. Chemical

Lettuce leaves have been obtained from a local Egyptian markets. CCl₄ was purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and solvents were purchased from Sigma chemical Co. (St. Louis, MO, USA).

2.2. Preparation of lettuce extract

Lettuce leaves were air-dried at 40 °C for 7 d. Ten grams of powdered leaves were extracted using 100 mL of 40% ethanol (plant:solvent, 1:10, w/v) under reflux for 30 min. After cooled, the extracts were filtered and evaporated under reduced pressure yielding a dry residue. The supernatant being the lettuce extract was decanted and kept at 4 °C until used.

2.3. Animals

This study was carried out on healthy male albino rats weighing 250–260 g. Seventy rats were obtained from the Central Animal House, Faculty of Veterinary Medicine, Zagazig University, Egypt. The animals were housed in conventional cages with free access to water and food at (21±2) °C with a 12 h light–dark cycle, 50% humidity and (25±3) °C. Rats received standard pellet diet comprised of 20% crude protein, 5% fat, 4.5% crude fiber, 8% ash, 2% calcium, 0.6% phosphorus, 3.4% glucose, 2% vitamins and 55% nitrogen free extract and water *ad libitum*.

2.4. Experimental design

Animals were divided into seven groups of 7 rats each. Treatment of rats with CCl₄ was carried out according to Gonzales *et al.* with some modifications to establish the reversible cirrhosis^[29]. Treatment of different groups was as follows.

Group I: remained untreated.

Group II: Olive oil (5 mL/kg body weight orally once a week) + DMSO (5 mL/kg body weight after 72 h of olive oil once a week) for 10 weeks.

Group III: lettuce extract (200 mg/kg body weight orally, dissolved in DMSO) once a week for 10 weeks.

Group IV: CCl₄ (2 mL/kg body weight in olive oil once a week, intraperitoneally) for 10 weeks.

Group V: CCl₄ (2 mL/kg body weight in olive oil once a week, intraperitoneally) for 10 weeks + lettuce extract (100 mg/kg body weight orally, dissolved in DMSO after 72 h of CCl₄ treatment) once a week for 10 weeks.

Group VI: CCl₄ (2 mL/kg body weight in olive oil once a week, intraperitoneally) for 10 weeks + lettuce extract (150 mg/kg body weight orally, dissolved in DMSO after 72 h of CCl₄ treatment) once a week for 10 weeks.

Group VII: CCl₄ (2 mL/kg body weight in olive oil once a week, intraperitoneally) for 10 weeks + lettuce extract (200 mg/kg body weight orally, dissolved in DMSO after 72 h of CCl₄ treatment) once a week for 10 weeks.

2.4.1. Preparation of serum, plasma and tissue homogenate

At the end of the experimental period, the animals were sacrificed by cervical decapitation. Blood was collected and centrifuged for serum separation. For plasma, blood was collected with anticoagulant and centrifuged (2000 g for 20 min) to separate plasma. The tissues were dissected out, weighed and washed using ice cold saline solution. Tissues were minced and homogenized (10% w/v) in Tris–HCl buffer (0.1 mol/L; pH 7.4) and centrifuged at 3000g for 20 min at 4 °C. The resulting supernatant was used for various biochemical assays.

2.5. Determination of body weights

Body weight of all animals in each group was recorded and is considered as the initial body weight. At the end of experiment after 10 weeks before dissection, body weight was again

recorded for all animals in each group. Percent increase in body weight was calculated as compared to the initial weight for all animals.

2.6. Assessment of testis size

At the end of the 10–week experiment, abdomen of each rat was incised to remove both the testis. Each testis was then washed with normal saline to separate the surrounding fat and connective tissues. After drying the surface with filter paper, weight of the testis was recorded and average was determined for each animal. Relative testis weight was calculated as the weight of testis to that of the final body weight for each rat.

2.7. Histopathology of testis and liver

The left testicle of each rat was serially sectioned and fixed in Bouin solution for 48 h. The specimens were then dehydrated through a gradual series of alcohol and cleared in three changes of xylene before embedded in paraffin. Serial sections, each of 4 µm thickness, were made and stained with hematoxylin and eosin according to standard method. Histological assessment was performed under light microscope in terms of the changes in different groups as compared to control group. Likewise the histology of liver from the CCl₄ group of rats was also performed.

2.8. Biochemical studies

About 100 mg of testis (right) tissue was homogenized in 10 volume of 100 mmol/L KH₂PO₄ buffer containing 1 mmol/L ethylene diamine tetraacetic acid (EDTA), pH 7.4 and centrifuged at 12000 g for 30 min at 4 °C. The supernatant was collected and used for the following experiments as described below. Protein concentration of the supernatant of testis tissue was determined by using crystalline bull serum albumin as standard.

2.8.1. CAT activity

The enzyme CAT catalyzes the conversion of H₂O₂ into water. A volume 3 mL of CAT reaction solution contained 50 mmol/L phosphate buffer (pH 7.0), 5.9 mmol/L H₂O₂, and 0.1 mL of enzyme extract. The reaction was initiated by adding the enzyme extract. Changes in absorbance of the reaction solution at 240 nm were noted after every 30 s. One unit CAT activity was defined as an absorbance change of 0.01 unit per min.

2.8.2. SOD activity

The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). The reaction solution (3 mL) contained 50 µmol/L NBT, 1.3 µmol/L riboflavin, 13 mmol/L methionine, 75 nmol/L EDTA, 50 mmol/L phosphate buffer (pH 7.8), 20–50 µL of enzyme extract. The test tubes containing the reaction solution were irradiated under a light (15 W florescent lamps) at 78 µmol/m²/s for 15 min. The absorbance of solution was noted at 650 nm. One unit of SOD activity was defined

as the amount of enzyme, which caused 50% inhibition of photochemical reduction of NBT.

2.8.3. Peroxidase (POD) activity

The POD activity was assayed by using the guaiacol oxidation method. The 3 mL POD reaction solution contained 50 mmol/L phosphate buffer (pH 5.0), 20 mmol/L guaiacol, 40 mmol/L H₂O₂, and 0.1 mL of enzyme extract. Changes in absorbance of the reaction solution at 470 nm were noted after every 20 s. One unit POD activity was defined as an absorbance change of 0.01 units/min.

2.8.4. Reduced glutathione (GSH)

GSH content in liver was measured spectrophotometrically by using Ellman's reagent as a coloring reagent. The absorbance was read at 412 nm with a spectrophotometer. A standard graph was drawn using different concentrations of a standard GSH solution and GSH contents were calculated as nmol/L per mg of tissue protein.

2.8.5. Glutathione peroxidase (GSH-Px) activity

Glutathione peroxidase activity was assayed by the method of Mohandas *et al*^[30]. The reaction mixture consisted of 1.49 mL phosphate buffer (0.1 mol/L, pH 7.4), 0.1 mL EDTA (1 mmol/L), 0.1 mL sodium azide (1 mmol/L), 0.05 mL glutathione reductase (1 μU/mL), 0.05 mL GSH (1 mmol/L), 0.1 mL NADPH (0.2 mmol/L), 0.01 mL H₂O₂ (0.25 mmol/L) and 0.1 mL 10% PMS in a total volume of 2 mL. The disappearance of NADPH at 340 nm was recorded at 25 °C. Enzyme activity was calculated as μmol/L NADPH oxidized/min/mg protein using molar extinction coefficient of 6.22×10³ M/cm.

2.8.6. Thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation level in the liver was measured as thiobarbituric acid reactive substances (TBARS). The absorbance was read at 532 nm. The concentration of TBARS was expressed as μg of TBARS per mg of tissue using 1,1,3,3-tetramethoxypropane (TMP) as standard.

2.8.7. Estimation of nitrite level

Nitrite assay was conducted by using Griess reagent. Five hundred microlitre of Griess reagent (1:1 solution

of 1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylamine dihydrochloric acid in water) was added to 100 μL of deproteinated homogenate and absorbance was measured at 546 nm. Nitrite concentration was calculated using a standard curve for sodium nitrite.

2.9. Serum level of hormones

Serum level of testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH), estradiol and prolactin was estimated by methods of Santos AM, *et al*^[31]. Standards, samples and controls were analyzed in duplicate in a single batch. In all assays, the coefficient of intra- and inter-assay variability was below 10%.

2.10. Statistical analysis

Parametric data, expressed as mean and standard error of the mean (SEM), were analyzed through one way ANOVA, followed by the *post hoc* Fisher least significant difference for comparison of various treatments using the SPSS 13.0. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Body and testis weights

As shown in Table 1, the treatment of male rats with CCl₄ for 10 weeks caused a significant ($P < 0.0001$) loss in the final body, testicular weights ($P < 0.0001$) and relative testis weights (representing the ratio between the mean body and testicular weights) compared with the control group. In the CCl₄+lettuce extract-treated groups the percent increase in body, testicular and relative testicular weights increased in a dose dependent manner (Table 1). At the lowest dose of lettuce extract (100 mg/kg bw), percent increase in body, testicular and relative testicular weights were statistically not different from the CCl₄-treated group. By contrast, body, testicular and relative testicular weights were increased significantly ($P < 0.01$ or $P < 0.0001$) at higher doses of lettuce extract as compared to the CCl₄-treated group. Administration of lettuce extract only did not change the

Table 1

Protective role of lettuce extract on body and testicular weight (Mean±SE, n=7).

Groups	Treatment	Initial body weight (g)	Final body weight (g)	% increase	Absolute testis weight (mg)	Relative testis weight as (%) of body weight
I	Control	266.50±2.83	373.00±2.99	40.61±1.84 ^b	2032.10±18.60 ^b	0.540±0.006 ^b
II	Vehicle	268.90±2.73	372.20±2.54	38.16±1.66 ^b	2028.50±20.70 ^b	0.540±0.006 ^b
III	lettuce extract 200 mg/kg	267.40±3.41	374.00±2.70	39.86±2.31 ^b	2083.30±22.50 ^b	0.550±0.008 ^b
IV	CCl ₄ 2 mL/kg	271.70±2.26	338.00±2.50	24.47±1.35 ^b	1468.40±14.00 ^b	0.420±0.003 ^b
V	CCl ₄ +lettuce extract 100 mg/kg	266.80±2.45	347.70±2.86	30.35±1.34 ^b	1510.80±16.70 ^b	0.420±0.006 ^b
VI	CCl ₄ +lettuce extract 150 mg/kg	264.20±1.92	354.10±3.60	34.10±1.84 ^{ab}	1654.80±24.20 ^b	0.460±0.006 ^{ba}
VII	CCl ₄ +lettuce extract 200 mg/kg	266.50±2.72	374.50±2.80	40.65±1.71 ^b	1880.30±26.70 ^b	0.490±0.009 ^{bb}

Significance from control group A, $P < 0.01$, B, $P < 0.0001$.

Significance from CCl₄ group a, $P < 0.01$, b, $P < 0.0001$.

final body weight, testicular and relative testicular weights as compared with that in the control group.

3.2. Effect of lettuce extract on testicular histopathology

There were no marked changes in testicular histology relative to controls in the vehicle as well as lettuce extract treated groups. Thus, normal spermatogenesis, well preserved Sertoli cells and well delineated tubular basement membrane were observed in Group I, Group II and Group III. The interstitium between tubules and Leydig cells was also intact. However, in the CCl_4 -treated group, differences were observed in histology of testis. Complete atrophy of seminiferous tubules was exhibited while in other areas of the section the tubular basement membranes of seminiferous tubules were identified, but most of the germ cells were degenerated, especially the ones involving highly differentiated germ cells along with deformed sperms. Partially the ground substance within the interstitium also disappeared and replaced by fibroblast and inflammatory cells. In the CCl_4 +lettuce extract-treated groups, toxic effects were ameliorated in a dose dependent manner (Figure 1).

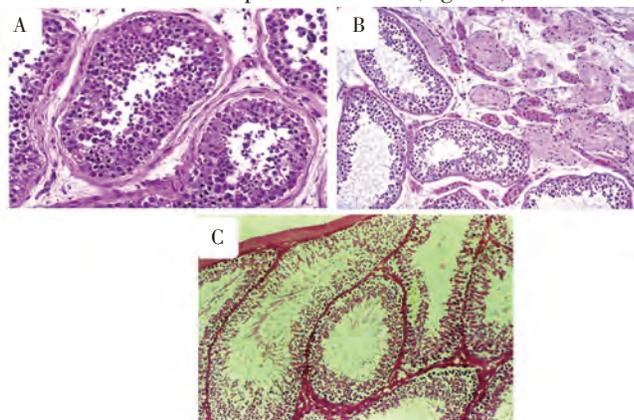


Figure 1. Effect of lettuce extract on testicular histopathology.

A: Section from testis of control rat showing intact seminiferous tubules. B: Section from testis of CCl_4 -treated rat showing atrophy of seminiferous tubules, infiltration of fibroblast and degeneration of basement membrane. C: Section from testis of CCl_4 +lettuce extract 200 mg/kg showing the germinal layers and presence of basement membrane.

3.3. Affirmation of cirrhosis in liver

Thin sections of liver from the CCl_4 treated group of rat showed

the centrilobular necrosis, fatty degeneration of hepatocytes, constriction of capillaries and structural disruption of the lobule (Figure 2).

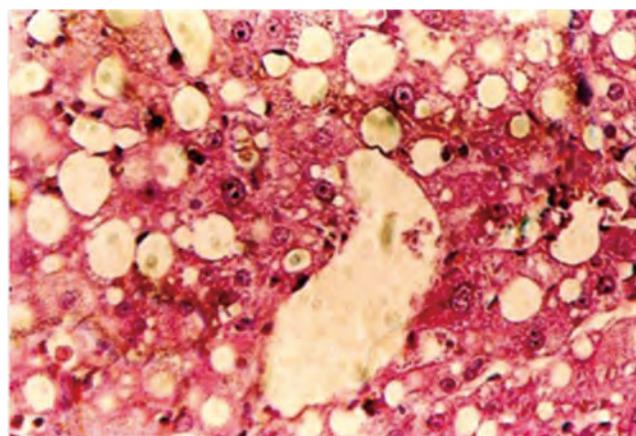


Figure 2. Representative section of liver from CCl_4 -treated group of rat showing centrilobular necrosis, fatty degeneration and structural disruption of the lobule (H & E stain).

3.4. Effect of lettuce extract on CCl_4 -induced changes in the enzymes activity

Effects of the lettuce extract on CAT, POD, SOD activity for all experimental groups are given in Table 2. Treatment of rats with CCl_4 significantly decreased the activities of CAT, POD, SOD and GSH-Px enzymes in testis tissue homogenates as compared to the control group. This reduction was improved significantly by the treatment with lettuce extract and the enzyme activities were increased as compared to the CCl_4 -treated group in a dose dependent manner.

3.5. Effect of lettuce extract on lipid peroxidation

The results are shown in Table 3. TBARS contents were increased while reduced glutathione (GSH) level decreased significantly in CCl_4 -induced toxicity group against the control group. Treatment with lettuce extract erased the effects of CCl_4 intoxication and TBARS contents decreased whereas GSH level was significantly increased in the testis homogenate.

3.6. Effect of lettuce extract on nitrite concentration

Carbon tetrachloride treatment for 10 weeks significantly

Table 2

Protective role of lettuce extract on antioxidant enzymes (Mean±SE, n=7).

Groups	Treatment	SOD (U/mg protein)	CAT (U/min)	POD (U/min)	GSH-Px (μmol/L/mg protein)
I	Control	89.9±1.1	4.30±0.13	3.18±0.13	23.16±0.65
II	Vehicle	90.7±2.6	4.00±0.12	3.19±0.11	23.10±0.77
III	lettuce extract 200 mg/kg	86.2±1.1	3.90±0.13	3.15±0.14	22.50±0.65
IV	CCl_4 2 mL/kg	43.1±1.6	1.80±0.13	1.50±0.06	13.28±0.46
V	CCl_4 + lettuce extract 100 mg/kg	50.7±1.4	2.10±0.10	1.94±0.10	15.87±0.46
VI	CCl_4 + lettuce extract 150 mg/kg	64.7±1.7	2.80±0.13	2.38±0.11	17.87±0.51
VII	CCl_4 + lettuce extract 200 mg/kg	79.1±2.0	3.80±0.16	2.93±0.11	21.04±0.66

Significance from control group A, $P<0.05$, B, $P<0.01$, C, $P<0.0001$.

Significance from CCl_4 group a, $P<0.01$, b, $P<0.0001$.

increased nitrite concentration in testis tissue homogenate as compared with the control group. Administration of lettuce extract ameliorated the effects of CCl₄ toxicity and significantly decreased nitrite contents in a dose dependent manner (Table 3).

3.7. Effect of lettuce extract on pituitary–gonadal axis

The mean values of the serum hormones, testosterone, luteinizing hormone and follicle stimulating hormone are shown in Table 4. After treatment of rats with CCl₄ for 10 weeks, the mean values of testosterone ($P<0.01$), luteinizing hormone ($P<0.0001$) and follicle stimulating hormone ($P<0.0001$) were decreased as against the control group. In the CCl₄+lettuce extract treated groups, the level of testosterone, luteinizing hormone and follicle stimulating hormone was increased in a dose dependent manner. Testosterone level did not change at lower dose of lettuce extract (100 mg/kg bw), however, it was increased significantly ($P<0.0001$) at the higher doses of lettuce extract (150 and 200 mg/kg body weight) as compared to the CCl₄–treated group. Serum level of luteinizing hormone with lettuce extract treatment did not differ from the CCl₄–treated group.

Mean values of follicle stimulating hormone did not change at the doses of 100 and 150 mg/kg body weight, by contrast, it was increased significantly at the dose of 200 mg/kg body weight compared with that in the CCl₄–treated group. Although an increase in the mean values of testosterone, luteinizing hormone and follicle stimulating hormone in the lettuce extract treatment alone occurred, it was not statistically significant compared with the control group.

After the treatment of rats with CCl₄ for 10 weeks the mean values of estradiol and prolactin increased significantly ($P<0.01$) as compared to that of control group (Table 4). Lettuce extract treatment co-administered with CCl₄ for 10 weeks lowered the mean values of estradiol and prolactin in a dose dependent manner. At the lower dose of lettuce extract (100 mg/kg body weight) the mean values of estradiol and prolactin did not change ($P>0.05$), while the mean values were increased significantly ($P<0.05$ and $P<0.01$) at the higher doses of lettuce extract, *i.e.*, 150 and 200 mg/kg body weight, respectively. In the lettuce extract group of rats, the mean values of estradiol and prolactin were reduced, but these were not statistically significant ($P>0.05$) as compared with the control group.

Table 3

Protective role of lettuce extract on lipid peroxidation and nitrite (Mean±SE, n=7).

Groups	Treatment	TBARS µg/mg protein	GSH µmol/L/mg protein	Nitrite µmol/L/mg protein
I	Control	35.4±1.05	30.2±1.13	74.7±0.86
II	Vehicle	34.8±1.13	29.5±1.43	74.0±0.76
III	lettuce extract 200 mg/kg	35.8±1.25	30.2±1.41	72.5±0.82
IV	CCl ₄ 2 mL/kg	60.5±2.30	13.1±1.12	99.0±1.54
V	CCl ₄ +lettuce extract 100 mg/kg	55.3±1.80	17.1±1.03	96.2±1.53
VI	CCl ₄ +lettuce extract 150 mg/kg	44.6±2.16	20.1±1.15	86.5±1.10
VII	CCl ₄ +lettuce extract 200 mg/kg	37.9±1.72	23.9±1.46	77.8±1.44

Significance from control group A, $P<0.05$, B, $P<0.0001$.

Significance from CCl₄ group a, $P<0.01$, b, $P<0.0001$.

Table 4

Protective role of lettuce extract on reproductive hormones (Mean±SE, n=10).

Groups	Treatments	Testosterone (ng/mL)	Luteinizing hormone (ng/mL)	Follicle stimulating hormone (ng/mL)	Estradiol (ng/mL)	Prolactin (ng/mL)
I	Control	3.11±0.16	1.76±0.10	20.03±0.34	13.07±0.14	23.06±0.19
II	Vehicle	3.16±0.08	1.74±0.12	19.98±0.44	13.28±0.22	23.00±0.31
III	lettuce extract 200 mg/kg	3.31±0.07	1.79±0.07	21.15±0.36	12.79±0.18	22.81±0.23
IV	CCl ₄ 2 mL/kg	1.50±0.09	1.16±0.07	17.12±0.24	18.33±0.18	28.24±0.45
V	CCl ₄ + lettuce extract 100 mg/kg	1.66±0.08	1.21±0.03	17.43±0.20	17.59±0.18	27.58±0.61
VI	CCl ₄ + lettuce extract 150 mg/kg	1.88±0.06	1.22±0.02	17.64±0.21	16.80±1.19	26.20±0.34
VII	CCl ₄ + lettuce extract 200 mg/kg	2.28±0.08	1.33±0.03	18.00±0.24	15.74±0.98	24.45±0.37

Significance from control group A, $P<0.05$, B, $P<0.0001$.

Significance from CCl₄ group a, $P<0.01$, b, $P<0.0001$.

4. Discussion

Our study, focused on investigating the effects of lettuce extract against CCl₄–induced toxicity in rats, showed that lettuce extract played an important role as a protective factor for CCl₄–induced toxicity. A number of chemicals including various environmental toxicants and even clinically useful drugs can

cause severe cellular damages in different organs of the body through metabolic activation to highly reactive substances such as free radicals[32].

CCl₄–induced toxicity as experimental models was well characterized. It has been extensively used for inducing free radical damages. It is bioactivated by cytochrome–P450 into free radicals leading to deleterious effects on liver due to lipid

peroxidation^[33]. From the present study, it has been observed that CCl₄-induced significant decrease in CAT, POD, SOD, and GSH-Px activities, depleted the GSH contents and enhanced lipid peroxidation (TBARS) in testis. It has been reported that CAT, POD, SOD and GSH-Px constitute a mutually supportive team of defense against ROS. Because of the low activity of antioxidant enzymes in the testis and decreased content of GSH, the CCl₄-induced oxidative stress turn to severe; the inbuilt mechanism of body fails to alleviate the damage. The decreased activity of SOD in testis in CCl₄-treated rats may be due to the enhanced lipid peroxidation or inactivation of the antioxidant enzymes. Decrease in GSH-Px activity during CCl₄ toxicity might be due to the decreased availability of GSH resulted during the enhanced lipid peroxidation. Lin *et al.* studied the effects of CCl₄ on lipid peroxidation and enzyme activities of SOD, CAT and GSH-Px^[1]. They observed increased lipid peroxidation in liver and kidneys, while antioxidant enzyme activities were decreased in these organs similar to the present study. Co-administration of lettuce extract ameliorated the decrease in the activities and levels of antioxidant enzymes CAT, POD, SOD, GSH-Px, the reduction in the GSH levels and the increase in lipid peroxidation in testis. It is possible that the antioxidant properties of the lettuce extract could be due to the presence of flavonoids and saponins in the extract.

CCl₄-induced testis injuries have been associated with enhanced nitrite production. Nitric oxide (NO) is a water- and lipid-soluble free radical synthesized in the vascular endothelium from L-arginine by the action of NO synthase enzymes. It plays an important role in the regulation of blood flow in normal and pathologic situations. There could be two reasons for the elevation of tissue NO levels after exposure to CCl₄-treatment: increased synthesis because of the injury of the vascular endothelium, or the activation of neutrophils in damaged testicular tissue, leading to the synthesis of NO. Muriel reported that nitrite is produced in the liver of treated rats with CCl₄^[34]. Nitrites can turn into NO in acidic pH. Peroxynitrite anions have been generated by the reaction of NO and superoxide anion. These peroxynitrite anions oxidize biomolecules, which finally lead to lipid peroxidation observed increased level of nitrite in liver and kidneys during chronic administration of CCl₄^[35]. Unilateral testicular torsion of rats resulted in higher level of nitrite contents in the testicular tissues^[36]. Oral treatment of rats with dimethyl ether decreased the nitrite level in this study. Scavenging ability of lettuce extract may be involved in this protective effect against CCl₄ toxicity. This plant contains the active constituents (flavonoids and saponins) which directly or indirectly scavenge the oxidative damage to different cells and organs while normalizing their function.

During the present study, low dose of CCl₄ was administered for a longer period of time to establish the liver cirrhosis in rat. Intraperitoneal administration of CCl₄ to rats at a lower dose and for longer period of time allowed the development of liver cirrhosis in all the treated animals^[29]. Exposure of rats to CCl₄ in this study indicated the disruption of lobules and hepatocyte

injuries, *i.e.*, including structural disruption of the lobule, centrilobular necrosis, fatty degeneration and constriction of capillaries. Different reactive compounds generated through CCl₄ metabolism singly *per se* is not considered the ultimate cause of CCl₄-induced cell death; it is by cooperation that they achieve a fatal outcome, provided the toxicant acts in a high single dose, or over longer periods of time at low doses. The dose of CCl₄ (2 mL/kg body weight once a week for 10 weeks) administered to rats in this study is found optimal for inducing reversible cirrhosis.

Spermatogenesis is a sophisticated and complex differentiation process. Degeneration of spermatogenesis is an integral and important part of normal spermatogenesis. However, spermatogonial degeneration can result from exposure to toxic chemicals. The toxic effects of CCl₄ on testicular histology were confirmed. In the CCl₄-treated group seminiferous tubules and germ cells were degenerated, interstitium partially disappeared and replaced by fibroblast and inflammatory cells in some of the areas of testis. Presence of saponins and flavonoids in lettuce extract might be involved in ameliorating the effects of CCl₄-induced toxicity and concomitantly near to normal histology was seen in lettuce extract treated groups.

The successful and complete male germ cell development is dependent on the balanced endocrine interplay of hypothalamus, pituitary and the testis. Gonadotropin releasing hormone secreted by the hypothalamus elicits the release of gonadotropins, *i.e.*, FSH and LH from the pituitary gland. FSH binds with receptors in the Sertoli cells and stimulates spermatogenesis. LH stimulates the production of testosterone in Leydig cells, which in turn may act on the Sertoli cells and peritubular cells of the seminiferous tubules and stimulates spermatogenesis^[37]. In the present study, CCl₄ treatment decreased the serum level of testosterone, FSH and LH. Secretion of testosterone is probably impaired due to excessive oxidative stress and the degeneration of Leydig cells^[31]. Metabolites of the testosterone reciprocally depress FSH and LH secretion. Injuries in germinal epithelial caused with CCl₄ treatment can stimulate spermatogenesis partially that may occur due to less production of androgen binding proteins. Toxic effects of CCl₄ may result in the failure of pituitary to secrete FSH and LH and that will result in testicular dysfunction leading to infertility.

In the present study, estradiol and prolactin serum level appeared to be positively associated in the control and all the treated groups but probably the direct stimulation of the pituitary by estradiol is the only one of the factors determining prolactinemia, with hypothalamic dysfunction being associated, as observed in hypogonadism. This mechanism may be considered partly responsible for the central origin of hypogonadism in our study.

Treatment of rats with CCl₄+lettuce extract ameliorated the toxic effects of CCl₄ and the level of testosterone, FSH and LH were increased, by contrast the serum level of estradiol and prolactin was decreased in a dose dependent manner. This plant contains the active constituents (flavonoids and saponins) which directly or indirectly scavenge the oxidative damage to different

cells and organs while normalizing their function. In addition, treatment of rats with lettuce extract alone, slightly increases the serum level of testosterone, FSH and LH, while reduce the level of estradiol and prolactin. It might be considered that aside from its scavenging activity it might directly vitalize the central nervous system. Our data suggest that the ability of lettuce extract to ameliorate CCl₄-induced testis injury is associated with its antioxidant and ROS scavenging properties. Our data also support the use of lettuce (*L. sativa*) in secondary infertility.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This research was funded by Faculty of Agriculture, Zagazig University (Project Number 34–2012).

Comments

Background

Liver is the vital organ which metabolises most of the drugs and chemicals, it plays important role in the detoxification of chemicals and drugs. Liver is the first organ to metabolize foreign compounds and hence it is susceptible to many different diseases. Some diseases are rare but a few, including hepatitis, cirrhosis, alcohol-related disorders and liver cancer are common. Changing lifestyle is increasing liver injury and causes altered liver functions. Therefore, there is need of new and potent hepatoprotective agents all over the world.

Research frontiers

Treatment of rats with CCl₄+*L. sativa* (lettuce extract) ameliorated the toxic effects of CCl₄ and the level of testosterone, FSH and LH were increased, by contrast the serum levels of estradiol and prolactin were decreased in a dose dependent manner. *L. sativa* contains the active constituents (flavonoids and saponins) which directly or indirectly scavenge the oxidative damage to different cells and organs while normalizing their function. In addition, treatment of rats with lettuce extract alone, slightly increases the serum level of testosterone, FSH and LH, while reduce the level of estradiol and prolactin. It might be considered that aside from its scavenging activity it might directly vitalize the central nervous system.

Related reports

CCl₄ was reported to cause hepatic necrosis due to formation of free radicals. The folklore medicine has

evidence of effectiveness of vegetables in treating various liver disorders.

Innovations & breakthroughs

The results suggest that the ability of lettuce extract to ameliorate CCl₄-induced testis injury is associated with its antioxidant and ROS scavenging properties. In the present study, authors have demonstrated the hepatoprotective activity of *L. sativa* in CCl₄ rat models.

Applications

From the literature review it has been found that *L. sativa* is a promising vegetable for humans. This scientific study supports and suggests the use of this plant as an adjuvant along with commonly used hepatoprotective agent. *L. sativa* was found to be a promising hepatoprotective agent in CCl₄ rat models. The results support the use of lettuce (*L. sativa*) in secondary infertility.

Peer review

This is a promising research work in which authors have demonstrated the hepatoprotective activity of *L. sativa* in CCl₄-induced liver damage in rats. The activity was assessed based on biochemical parameters, antioxidant enzyme levels in liver homogenate and histopathological observations. *L. sativa* was found to be a promising hepatoprotective agent in CCl₄ rat models.

References

- [1] Lin HM, Tseng HC, Wang CJ, Lo CW, Chou FP. Hepatoprotective effects of *Solanum nigrum* Linn extract against CCl₄-induced oxidative damage in rats. *Chem Biol Interact* 2008; **171**: 283–293.
- [2] Shaker E, Khaled RZ, Wajahat ZM, Gamal ES, Tarek MI. Comparison of imatinib, nilotinib and silymarin in the treatment of carbon tetrachloride-induced hepatic oxidative stress, injury and fibrosis. *Toxicol App Pharmacol* 2011; **252**: 165–175.
- [3] Shaker E, Mahmoud H, Mnaa S. Silymarin, the antioxidant component and *Silybum marianum* extracts prevent liver damage. *Food Chem Toxicol* 2010; **48**: 803–806.
- [4] Zhan L, Hu J, Ai Z, Pang L, Li Y, Zhu M. Light exposure during storage preserving soluble sugar and L-ascorbic acid content of minimally processed romaine lettuce (*Lactuca sativa* L. var. longifolia). *Food Chem* 2013; **136**: 273–278.
- [5] Liu Q, Baohua K, Li G, Ning L, Xiufang X. Hepatoprotective and antioxidant effects of porcine plasma protein hydrolysates on carbon tetrachloride-induced liver damage in rats. *Food Chem Toxicol* 2011; **49**: 1316–1321.
- [6] Adesanoye OA, Farombi EO. Hepatoprotective effects of *Vernonia amygdalina* (astereaceae) in rats treated with carbon tetrachloride. *Exp Toxicol Pathol* 2010; **62**: 197–206.
- [7] Blomhoff R. Dietary antioxidants and cardiovascular disease. *Curr Opin Lipidol* 2005; **16**: 47–54.
- [8] Shim JY, Kim MH, Kim HD, Ahn JY, Jie-Young S. Protective

- action of the immunomodulator ginsan against carbon tetrachloride-induced liver injury via control of oxidative stress and the inflammatory response. *Toxicol Appl Pharmacol* 2010; **242**: 318–325.
- [9] Qian ZJ, Jung WK, Kim SK. Free radical scavenging activity of a novel antioxidative peptide purified from hydrolysate of bullfrog skin, *Rana catesbeiana* shaw. *Bioresour Technol* 2008; **99**: 1690–1698.
- [10] Valko M, Leibfritz D, Moncol J, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 2007; **39**: 44–84.
- [11] Noyan T, Kömüroğlu U, Bayram I, Sekeroğlu MR. Comparison of the effects of melatonin and pentoxifylline on carbon tetrachloride-induced liver toxicity in mice. *Cell Biol Toxicol* 2006; **22**: 381–391.
- [12] Sanmugapriya E, Venkataraman S. Studies on hepatoprotective and antioxidant actions of *Strychnos potatorum* Linn. seeds on CCl₄-induced acute hepatic injury in experimental rats. *J Ethnopharmacol* 2006; **105**: 154–160.
- [13] Akram E, Maryam E, Al-Ebrahime M, Rohani AH, Mortazavi P. Protective effects of sodium molybdate on carbon tetrachloride-induced hepatotoxicity in rats. *J Trace Elem Med Biol* 2011; **25**: 67–71.
- [14] Lee KJ, Woo ER, Choi CY, Shin DW, Lee DG, You HJ, et al. Protective effect of acteoside on carbon tetrachloride-induced hepatotoxicity. *Life Sci* 2004; **74**: 1051–1064.
- [15] Arts IC, Hollman PC. Polyphenols and disease risk in epidemiologic studies. *Am J Clin Nutr* 2005; **81**: 317S–325S.
- [16] Seeram NP, Adams LS, Henning SM, Heber D. *In vitro* antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. *J Nutr Biochem* 2005; **16**: 360–367.
- [17] Middleton E, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev* 2000; **52**: 673–751.
- [18] Dupont S, Mondy Z, Willamson G, Price K. Effect of variety, processing, and storage on the flavonoid glycoside and composition of lettuce and chicory. *J Agric Food Chem* 2000; **48**: 3957–3964.
- [19] Mulabagal V, Ngouajio M, Nair A, Zhang Y, Gottumukkala AL, Nair MG. *In vitro* evaluation of red and green lettuce (*Lactuca sativa*) for functional food properties. *Food Chem* 2010; **118**: 300–306.
- [20] Msilini N, Oueslati S, Amdouni T, Chebbi M, Ksouri R, Lachaâl M, et al. Variability of phenolic content and antioxidant activity of two lettuce varieties under Fe deficiency. *J Sci Food Agric* 2013; **93**(8): 2016–2021.
- [21] Al Nomaani RS, Hossain MA, Weli AM, Al-Riyami Q, Al-Sabahi JN, Rahman SM. Chemical composition of essential oils and *in vitro* antioxidant activity of fresh and dry leaves crude extracts of medicinal plant of *Lactuca sativa* L. native to Sultanate of Oman. *Asian Pac J Trop Biomed* 2013; **3**(5): 353–357.
- [22] Llorach R, Martínez-Sánchez A, Tomás-Barberán FA, Gil MI, Ferreres F. Characterisation of polyphenols and antioxidant properties of five lettuce varieties and escarole. *Food Chem* 2008; **108**: 1028–1038.
- [23] Nicolle C, Cardinault N, Gueux E, Jaffrelo L, Rock E, Mazur A. Health effect of vegetable-based diet: Lettuce consumption improves cholesterol metabolism and antioxidant status in the rat. *Clin Nutr* 2004; **23**: 605–614.
- [24] Serafini M, Bugianesi R, Salucci M, Azzini E, Raguzzini A, Maiani G. Effect of acute ingestion of fresh and stored lettuce (*Lactuca sativa*) on plasma total antioxidant capacity and antioxidant levels in human subjects. *Br J Nutr* 2002; **88**: 615–623.
- [25] Chu Y, Sun J, Wu X, Liu RH. Antioxidant and antiproliferative activity of common vegetables. *J Agric Food Chem* 2002; **50**: 6910–6916.
- [26] Sayyah M, Hadidi N, Kamalinejad M. Analgesic and anti-inflammatory activity of *Lactuca sativa* seed extract in rats. *J Ethnopharmacol* 2004; **92**: 325–329.
- [27] Edziri HL, Smach MA, Ammar S, Mahjoub MA, Mighri Z, Aouni M, et al. Antioxidant, antibacterial, and antiviral effects of *Lactuca sativa* extracts. *Ind Crop Prod* 2011; **34**: 1182–1185.
- [28] Zhan L, Hu J, Ai Z, Pang L, Li Y, Zhu M. Light exposure during storage preserving soluble sugar and L-ascorbic acid content of minimally processed romaine lettuce (*Lactuca sativa* L. var. longifolia). *Food Chem* 2013; **136**: 273–278.
- [29] Gonzales PH, Rhoden CR, Luz C, Correa G, Oliveira MC. Male gonadal function, prolactin secretion and lactotroph population in an experimental model of cirrhosis. *Braz J Med Biol Res* 2007; **40**: 1383–1388.
- [30] Mohandas J, Marshal JJ, Duggin GG, Horvath JS, Tiller DJ. Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney. Possible implications in analgesic nephropathy. *Biochem Pharmacol* 1984; **33**(11): 1801–1807.
- [31] Santos AM, Ferraz MR, Teixeira CV, Sampaio FJ, da Fonte RC. Effects of undernutrition on serum and testicular testosterone levels and sexual function in adult rats. *Horm Met Res* 2004; **36**: 27–33.
- [32] Khan MR, Rizvi W, Khan GN, Khan RA, Shaheen S. Carbon tetrachloride-induced nephrotoxicity in rat: protective role of *Digera muricata*. *J Ethnopharmacol* 2009; **122**: 91–99.
- [33] Adewole SO, Salako AA, Doherty OW, Naicker T. Effect of melatonin on carbon tetrachloride-induced kidney injury in Wistar rats. *Afr J Biomed Res* 2007; **10**: 153–164.
- [34] Muriel P. Nitric oxide protection of rat liver from lipid peroxidation, collagen accumulation, and liver damage induced by carbon tetrachloride. *Biochem Pharmacol* 1998; **56**: 773–779.
- [35] Srinivasan M, Rukkumani R, Ram Sudheer A, Menon VP. Ferulic acid, a natural protector against carbon tetrachloride-induced toxicity. *Fundam Clin Pharmacol* 2005; **19**: 491–496.
- [36] Koltuksuz U, Irmak MK, Karaman A, Ozyurt H, Akyol O. Testicular nitric oxide levels after unilateral testicular torsion/detorsion in rats pretreated with caffeic acid phenethyl ester. *Urol Res* 2000; **28**: 360–363.
- [37] Conn PM. The molecular basis of gonadotropin releasing hormone action. *Endocr Rev* 1986; **7**(1): 3–10.